XL. STUDIES IN CREATININE AND URIC ACID METABOLISM.

II. INGESTION OF PROTEINS AND AMINO-ACIDS AND THE HOURLY ELIMINATION OF CREATININE AND URIC ACID.

By HARRY ZWARENSTEIN.

From the Departments of Physiology and Physiological Chemistry, University of Capetown.

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It is almost universally accepted that urinary creatinine is independent of the amount of protein ingested but that protein ingestion exerts a marked influence on the excretion of uric acid. No attempt has yet been made to explain the marked difference shown by these two endogenous catabolites.

According to Mares [1888] the increased uric acid excretion on protein ingestion is due to the nuclear disintegration occasioned by the work of digestion. Another theory ascribes the increase to stimulation of purine synthesis. Graham and Poulton [1913] suggest that part of the endogenous uric acid arises by synthesis from protein and carbohydrate and part from the metabolism of cell nuclei. The latter, they state, "represents the general wear and tear in the tissues and is not affected by the diet given."

The work of Lewis *et al.* [1918, 1923] and Gibson and Doisy [1923] indicates that the influence exerted by protein is due to a general stimulation of cellular metabolism by the amino-acids. They have also suggested that the effect of amino-acids is a parallel phenomenon to their specific dynamic action.

There seems to be no reason, however, why there should not be an increase in creatinine excretion as well as in the uric acid excretion if protein or amino-acid ingestion causes a general stimulation of the cellular metabolism. The work reported in this paper was initially undertaken with the intention of investigating the effect of amino-acids on both the creatinine and uric acid excretions and thus to repeat the experiments of Lewis and Doisy as their results seemed rather remarkable when looked at from the point of view indicated above in regard to a possible effect on creatinine elimination. The first experiments with amino-acids showed results so at variance with the results of the American workers that it was thought advisable to repeat also the experiments on the influence of protein ingestion.

It was shown by Mares and others that during the increased nitrogen excretion which follows a meal the increase in uric acid has a briefer duration than the increase in urea and occurs characteristically in the earlier hours of the hyperexcretory period, and further, that while the urea is maintained above the level of the abstinence period, the uric acid falls to its abstinence value or even below this. The urea excretion may be regarded as the index of amino-acid assimilation and consequently on the basis of the general stimulation theory one should not expect such a large discrepancy in the time relations of the excretions of urea and uric acid.

Various observers have recorded no effect on the hourly elimination of uric acid as the result of protein feeding [cf. Hammarsten, 1922].

Siven [1901] found that extractives of meat caused an increase in uric acid excretion while protein had no influence. Part of the excreted uric acid comes from the ingested purine bases which are directly oxidised to uric acid, and part originates from the body. Bürian and Schur [1900], Kaufmann and Mohr [1902], Pfeil [1903] and others found that purine-free food had no influence on the excretion of uric acid.

Hopkins and Hope [1898] found that after ingestion of 410 g. of egg-white and one pint of milk some increase in uric acid was obtained, but it was very small compared with that after a meat diet of approximately equal nitrogen value.

In passing, it may be mentioned that the results of Hopkins and Hope have been quoted in several text-books of Physiological Chemistry in such a way as to create a totally erroneous impression. It is stated that after a meal of bread and potatoes a marked increase in the hourly uric acid excretion is obtained, whereas an examination of this experiment in the paper of Hopkins and Hope shows that this effect was obtained on ingesting 200 to 300 g. of meat and small quantities of bread and potatoes.

Experimental.

The subjects of these experiments were normal young men. For three or four days before the experimental day the diet was free from creatine, creatinine and purines. After the evening meal on the day before the experiment no food was taken until the end of the experiment except for the amino-acid or protein whose influence was being investigated. As a basis for comparison experiments in which no foodstuff was ingested were made and taken as fasting normals. 200 cc. of water were drunk every hour to ensure complete emptying of the bladder, and the creatinine and uric acid were estimated directly after collection.

Creatinine was determined by the Folin colorimetric method and uric acid by the colorimetric method of Benedict and Franke [1922]. For the uric acid estimation the urine was in every case so diluted that the reading for the "unknown" was always between 14 and 26; in the majority of cases it was between 17 and 23, the standard being set at 20 mm. Identical results were obtained for all subjects and except for the experiment with egg-white all the

results below are those for subject *A* who found great difficulty in ingesting a sufficient amount of egg-white.

Results and discussion.

The creatinine excretion was not affected by either protein or amino-acid ingestion, which is in agreement with the well-established fact that urinary creatinine is independent of the amount of protein in the diet. We can thus confine ourselves almost entirely to an examination and discussion of the uric acid figures, which are quite contrary to fairly widely accepted conclusions.

Examination of Tables III and IV shows very definitely that the ingestion of 10 or 20 g. of glycine or alanine had no effect on the hourly level of uric acid elimination, the glycine and alanine curves being almost identical with the fasting normal. The normal fasting uric acid level shows a decline during the afternoon hours, which is in accordance with the findings of numerous observers.

Table I. *Fasting normal. Subject A. Wt.* 53·6 kg.

Time	Vol. of urine	Creatinine	Uric acid	Remarks
Nov. 14th				
8– 9 a.m.	73 cc.	69·7 mg.	19·7 mg.	200 cc. water every hour (and in all experiments)
9–10	396	68·0	18·6	
10–11	576	65·5	21·4	
11–12	352	61·2	16·5	
12–1 p.m.	170	62·1	16·2	
1–2	158	66·8	14·7	
2–3	246	68·0	14·8	
3–4	180	69·0	12·0	
4–5	228	66·1	12·0	
Nov. 29th				
8– 9 a.m.	35 cc.	70·1 mg.	17·0 mg.	
9–10	268	69·0	17·6	
10–11	184	68·9	16·1	
11–12	188	73·7	16·6	
12– 1 p.m.	152	72·6	16·9	
1– 2	212	69·7	17·3	
2– 3	254	67·8	17·2	
3– 4	240	69·[illegible]	16·8	

Table II. *Nov. 16th. Ingestion of glycine. Subject A.*

Time	Vol. of urine	Creatinine	Uric acid	Remarks
8– 9 a.m.	132 cc.	73·8 mg.	19·2 mg	
9–10	438	72·0	19·1	
10–11	142	73·1	18·9	10 g. glycine at 10 a.m.
11–12	465	76·4	18·2	
12– 1 p.m.	265	69·4	18·2	
1– 2	75	71·6	16·0	
2– 3	196	69·1	14·7	
3– 4	200	74·0	14·4	

Table III. *Nov. 25th. Ingestion of alanine. Subject A.*

Time	Vol. of urine	Creatinine	Uric acid	Remarks
8– 9 a.m.	32 cc.	75·7 mg.	18·5 mg.	
9–10	49	72·5	18·8	
10–11	79	75·4	17·2	20 g. alanine at 10 a.m.
11–12	240	74·9	16·7	
12– 1 p.m.	422	78·0	15·3	
1– 2	112	76·9	12·1	
2– 3	138	75·5	13·0	
3– 4	172	70·0	13·1	

Table IV. *Nov. 1st. Fasting normal. Subject B. Wt.* 54·3 kg.

Time	Vol. of urine	Creatinine	Uric acid	Remarks
7– 8 a.m.	58 cc.	68·7 mg.	18·6 mg.	200 cc. water every hour
8– 9	155	73·4	20·0	
9–10	244	67·0	21·3	
10–11	82	66·0	20·0	
11–12	235	68·8	18·7	
12– 1 p.m.	145	70·4	18·6	
1– 2	170	70·0	18·6	
2– 3	186	70·6	18·0	

Table V. *Nov. 8th. Ingestion of egg-white. Subject B.*

Time	Vol. of urine	Creatinine	Uric acid	Remarks
7– 8 a.m.	24 cc.	68·8 mg.	15·3 mg.	
8– 9	27	67·1	20·0	
9–10	137	72·4	18·7	
10–11	32	65·8	18·3	250 g. boiled egg-white =4·3 g. N.
11–12	32	69·6	21·6	
12– 1 p.m.	40	74·0	20·8	
1– 2	116	70·9	16·0	
2– 3	196	74·8	16·0	
3– 4	348	70·0	15·3	
4– 5	68	65·0	14·7	

Table VI. *Nov. 22nd. Ingestion of cheese. Subject A.*

Time	Vol. of urine	Creatinine	Uric acid	Urea	Remarks
8– 9 a.m.	22 cc.	68·5 mg.	16·3 mg.		
9–10	144	68·2	18·0	—	
10–11	178	72·7	16·8	—	200 g. Cheddar cheese, 10–10.20 = 7·52 g. N.
11–12	220	73·2	16·6	1·10 g.	
12– 1 p.m.	66	72·7	15·5	1·06	
1– 2	118	77·6	18·4	1·53	
2– 3	220	72·3	17·6	1·76	
3– 4	378	72·2	15·9	1·13	
4– 5	158	74·2	17·0	1·58	
5– 6	124	67·6	12·4	1·00	

Table VII. *Nov. 18th. Ingestion of meat. Subject A. Wt.* 53·6 kg.

Time	Vol. of urine	Creatinine	Uric acid	Remarks
8– 9 a.m.	37 cc.	73·0 mg.	18·5 mg.	
9–10	112	69·1	19·7	
10–11	190	73·0	20·1	165 g. fried beefsteak, 35 g. white bread = 4·85 g. N. } 10–10.20
11–12	270	84·0	27·5	
12– 1 p.m.	150	88·8	34·9	
1– 2	226	86·9	40·0	
2– 3	240	80·0	31·3	
3– 4	216	77·0	22·7	
4– 5	258	76·4	20·1	
5– 6	204	72·7	17·0	
6– 7	226	70·3	19·7	

The ingestion of 200 g. of Cheddar cheese (Table VI) or of 250 g. boiled egg-white (Table V) had also no influence on the uric acid excretion. The increased excretion of urea (Table VI) indicates that assimilation of large amounts of amino-acids had occurred and reached its maximum $3\frac{1}{2}$ hours after ingestion.

After meat ingestion, however, a large increase occurred both in the creatinine and uric acid excretions (Table VII). The uric acid rose from 19 mg.

to 40 mg. and the creatinine from 72 to 80 mg. This result is in marked contrast to that obtained on ingesting large amounts of purine-free protein, and is in essential agreement with the results of Hopkins and Hope [1898], Burns and Orr [1916] and numerous other investigators.

We cannot escape from the conclusion that a purine-free diet has, under the conditions of these experiments, no influence on the hourly elimination of uric acid or creatinine, and that the increase after the ingestion of meat can be ascribed to the presence of creatinine and uric acid or their immediate precursors in the ingesta.

The above conclusions in regard to the effect of a purine-free diet taken in conjunction with the fact that assimilation of a large amount of amino-acids takes place on ingestion of purine-free protein seems definitely to dispose of any theory of general cellular stimulation by amino-acids, and of Mares's theory that an increase in uric acid excretion after protein ingestion can be due to the increased work of digestion, and consequently disposes also of the necessity for formulating any theory in regard to the effect of purine-free protein on the excretion of uric acid.

The absence of any influence on uric acid and creatinine of a purine-free protein diet is in line with the results obtained after ingestion of amino-acids.

We thus come to the general conclusion that the elimination of both uric acid and creatinine is independent of the amount of purine- and creatine-creatinine-free protein in the diet. It was shown in a previous paper [1926] that the daily variations in urinary creatinine and uric acid on such a diet follow one another closely. The results in this paper indicate that these variations are not due to variations in the amount of protein ingested.

On the whole the results, which indicate a further general correlation between uric acid and creatinine, are more in accordance with our conception of endogenous metabolism than the results hitherto published, which show such an unaccountable discrepancy in the reactions of these two endogenous catabolites to the nature of the diet.

Amongst others, Höst [1919] has shown that the excretion of uric acid varies within limits with increase or decrease in the calorific value of the food. Some of Rose's [1921] experiments also indicate that the calorie content of the food is the important factor.

In cases of starvation or low carbohydrate diets or diabetes the amounts of both creatinine and uric acid in the urine decrease, so that the excretion of creatinine seems to be subject to the same influences as the excretion of uric acid. One of the factors in causing variations in uric acid and creatinine excretions may thus be the effect of differences in the calorie value of the diet on tissue metabolism which is of course quite a different conception from that of specific dynamic action.

SUMMARY.

1. Ingestion of glycine or alanine is without effect on the hourly output of uric acid or creatinine.

2. A purine-free protein meal (egg-white and cheese) is similarly without effect on the hourly excretion of uric acid or creatinine.

3. Ingestion of a meal rich in uric acid or creatinine precursors causes a large increase in the excretion of these two catabolites in the urine.

4. Both urinary uric acid and creatinine are thus independent of the creatine-creatinine- and purine-free protein content of a diet.

5. It is suggested that the calorific value of the diet may be an important factor affecting both uric acid and creatinine metabolism.

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XLI. THE VITAMIN B CONTENT OF MALT EXTRACT.

By ALFRED LOUIS BACHARACH and EDITH ALLCHORNE.

From the Glaxo Research Laboratory, 56, Osnaburgh Street, London, N.W. 1.

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Some years ago Cooper [1914] showed that some, but not all, malt extracts cured polyneuritis in pigeons. Randoin and Lecoq [1927] more recently replaced 65 % to 70 % of the sucrose in a vitamin B-free diet by malt extract, and were able with the modified diet to secure normal growth and reproduction in rats, and also to prevent and cure polyneuritis in pigeons. Stammers [1924] secured satisfactory though sub-maximal growth in rats on a basal diet devoid of vitamin B with a supplementary diet representing 8 g. of malt per animal per day. Southgate [1924] showed that "barley and malt contain water-soluble B, as judged by the effect on rats, in considerable and equal amounts." Finally, Harden and Zilva [1924] showed that "the vitamin B of barley is not affected by the malting process and is frequently present in the finished malt, but is absent from the culms."

In describing our own experiments we shall use the term vitamin B for brevity, without prejudice to the question of the number of constituents in the water-soluble growth-promoting antineuritic vitamin complex. We have throughout used the Norwegian albino rat, of the stock invariably used in this laboratory [Bacharach, 1926].

The experiments described below were made to find out whether vitamin B was normally present in commercial malt extracts, and, if so, what was the source.

In the first set of experiments animals were fed on a diet deficient in vitamin B, according to the technique described by Bacharach and Hartwell [1927]. The animals used were selected so as to secure litter control. The period of feeding the basal diet alone, before adding the daily dose of substance to be tested, was 20 days. The animals were 36 days old when put on to the basal diet, weighing (males) from 54 to 59 g. and (females) from 48 to 53 g.

Two groups of animals were used, one of 8 males and the other of 8 females, and all the animals were put on to experiment on the same day.

The following substances were tested.

A. A blend of wheat and barley flour, unmalted.

B. The same blend of wheat and barley flour after malting.

C. The malt extract made from this flour, containing approximately 78 % total solids.

The malt extract was fed in doses of 1·2, 1·4, 1·6, and 1·8 g., a preliminary experiment having shown that the curative dose lay somewhere in this neighbourhood.

By the courtesy of Mr Gordon Cummings and Mr Frank Marshall of the British Diamalt Co., Sawbridgeworth, who made the malt extract, we obtained the samples referred to in A, B and C above, and were informed that the weight ratio of cereal to extract was approximately 5 : 4. Accordingly, the animals on the cereal supplement were fed 1·5, 1·75, 2·0 and 2·25 g. To make sure that the whole of this cereal was consumed, it was made into a "pill" with some of the basal diet and a little olive oil, and was fed each morning before the basal diet was replenished.

The results of the above experiment are summarised in Table I.

Table I. *Change of weight in grams during* 18 *days on various supplementary rations of cereal material.*

		"Preliminary period," 29 days.				
Controls		Litter I, ♂ −13 (23 days)	Litter II, ♀ −4 (23 days)	Litter I, ♂ −8 (31 days)	Litter II, ♀ +7 (23 days)	Totals for different materials
Flour	1·5 g.	+16	—	—	—	81
	1·75	—	+11	—	—	
	2·0	—	—	+28	—	
	2·25	—	—	—	+26	
Malted flour	1·5 g.	+20	—	—	—	96
	1·75	—	+20	—	—	
	2·0	—	—	+28	—	
	2·25	—	—	—	+28	
Malt extract	1·2 g.	+29	—	—	—	153
	1·4	—	+41	—	—	
	1·6	—	—	+37	—	
	1·8	—	—	—	+46	
Totals for different dosages		65	72	93	100	

Notes on Table I.

1. It will be noticed that the increases in growth are regular. If they are summed horizontally or vertically they give consistent results. The average daily growth per animal per gram of supplementary ration is almost identical for the unmalted and the malted flour, but considerably greater for the malt extract; the average daily growth increase per animal in each of the four groups is a function of the dosage of supplementary ration.

2. The malt extract used was a potent source of vitamin B, approximately 1·3 g. per day appearing adequate to restore young rats on a vitamin B-deficient diet to normal growth.

3. The malt extract appears to give better growth than the corresponding amount of the malted cereal from which it was made. It is out of the question that the mere process of extraction should have increased the amount of vitamin and this apparent advantage must be due to one or both of two

causes. First, the 5 : 4 ratio of cereal to extract, on which the dosage was based, may be slightly biased in favour of the malt extract. Secondly, an "appetite" effect may come into play. It was certainly noticed that the animals on the malt extract consumed their supplementary ration with much greater avidity than did those on the malted or unmalted flours. The latter, after consuming their "pills," which contained 2 or 3 g. of oil and basal diet as well as the cereal, generally retired to the back of their cages and ate very little more of the basal diet. The former attacked the malt extract with enthusiasm, and consumed almost the whole of their basal diet.

4. There is no significant difference between the vitamin B contents of the malted and the unmalted flours. The slightly better growth given by the malted flour is almost certainly also an "appetite" effect.

5. There is no evidence that vitamin B is either produced or destroyed during malting.

It having been established that the testing of malted substances for vitamin B on the albino rat is susceptible of giving consistent results, a further series of experiments was undertaken with four commercial brands of malt extract, of which the samples were bought in the open market except extract *M* which came from the same source as that used in the first set of experiments.

In this case three groups of animals were used, two groups of 5 males each and one group of 5 males and 5 females, all the animals from each group being from one litter; these we shall refer to as groups I, II and III respectively.

The technique used in these tests was identical with that used in the series of experiments described above, but the supplementary ration given was in all cases 1·0 g. per animal per day: the preliminary period on basal diet alone was 21 days. Groups I and II were put on experiment at the same time, and group III a week later. The results are summarised in Table II. The animals were 38 (groups I and II) and 34 (group III) days old when put on to the basal diet, weighing (males) from 40 to 53 g. and (females) from 42 to 51 g.

Table II. *Change in weight in grams during* 35 *days on* 1·0 *g. per day of malt extract.*

	Group I	Group II	Group III		
	♂	♂	♂	♀	Average
Extract *M*	+50	+49	+40	+34	43
Extract *T*	+50	+37	+20	+25	33
Extract *K*	+21	+24	+38	+30	28
Extract *B*	+41	+35	+ 3	+23	26
Controls	− 9 (37 days)	+ 1 (49 days)	−11 (33 days)	−13 (31 days)	

Notes on Table II.

1. The animals placed on experiment at the same time gave consistent results.

2. There is a discrepancy with extracts *B* and *K* between their effects on groups I and II on the one hand and on group III on the other.

3. The control animal in group II behaved abnormally, in that its weight increased from 50 to 76 g. over a period of 26 days. It then declined rapidly, and at the end of 49 days had reached its original weight, was in a very poor condition and had to be killed. The other three control animals behaved normally, showing very little increase in weight and then declining rapidly until they were moribund or dead.

4. On the average, extract *M* showed itself definitely to be possessed of the highest growth-promoting power; in all the groups extract *M* was the best source of vitamin B and extract *T* came second, except in group I where they were equal.

5. Extracts *B* and *K* are also active, though less so, but their comparative activity is difficult to assess owing to the complication mentioned in 2 above. No explanation has been found for this complication.

It is of interest to note that extract *M* was a brand which was known by previous examination to have a higher diastatic power than any other make examined.

Summary.

1. Several brands of commercial malt extract have been found to be rich sources of that vitamin which will restore to normal growth rats on a diet devoid of water-soluble growth-promoting factors.

2. The vitamin is derived from the unmalted flour.

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XLII. THE CHEMICAL ACTION OF QUINONES ON PROTEINS AND AMINO-ACIDS.

By EVELYN ASHLEY COOPER
and RAYMOND BENNETT HAINES.

From the Chemical Department, University of Birmingham.

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The previous work on this subject [Morgan and Cooper, 1924; Cooper and Nicholas, 1927] has shown that whilst *p*-benzoquinone is from 4 to 16 times as active a germicide as toluquinone, yet very little difference exists in their apparent chemical reactivity with such proteins as gelatin and albumin. With certain amino-acids, however, preliminary experiments showed that benzoquinone reacted 5 times as readily as toluquinone, thus suggesting that the germicidal action of the quinones is associated with their reactivity towards the free amino-acids present in the organism rather than with the cell-proteins. Further investigations have since been carried out in connection with this problem in order to substantiate, if possible, the earlier conclusions.

It was found [Cooper and Nicholas, 1927] that the quinones were unstable in aqueous solution when exposed to daylight, but underwent little or no change in the dark. The following results show that the process of decomposition is, furthermore, accompanied by a fall in bactericidal power. The quinones were estimated by the method of Valeur and germicidal power was determined either by the Chick-Martin method or by the inhibitory process.

Table I.

	Period of exposure to daylight	Concentration of solution (g. per litre)	Bactericidal concentration (*B. coli communis*)
Benzoquinone	Initial	0·1147	1 in 35,000
	2 days	0·1098	—
	13 „	0·0803	1 in 27,500
	27 „	0·0679	1 in 17,500
	47 „	0·0604	1 in 12,500
Toluquinone	Initial	0·0986	1 in 9,000
	1 day	0·0766	1 in 4,500
	4 days	0·0515	1 in 4,500
	7 „	0·0263	1 in 2,000

The results show that the rate of decomposition and fall in germicidal power is more pronounced with toluquinone than with benzoquinone. The chemical changes occurring in solutions of quinones are of great complexity; apparently reduction, oxidation, and polymerisation are involved.

The foregoing observations indicated the importance of careful control work. In fact, when prolonged experiments were in progress, the solutions were stored in darkness to avoid as far as possible loss of quinone.

Reference has already been made to the fact that benzoquinone may be as much as 4–16 times as efficacious as toluquinone in germicidal power. Some further determinations have since been made, using organisms that had not been previously employed. The results are set out in Table II.

Table II.

Organism	Conditions	Carbolic coefficients		
		Benzoquinone	Toluquinone	Ratio
B. typhosus	½ hr. disinfection at 20°	204	23	9
	1 ,, ,, ,, 20°	1400	83	17
B. coli	½ ,, ,, ,, 20°	268	52	5
	1 ,, ,, ,, 37°	6650	1710	4
B. pyocyaneus	½ ,, ,, ,, 20°	15·6	4·3	4
B. mesentericus	½ ,, ,, ,, 20°	230	21	11
B. prodigiosus	½ ,, ,, ,, 20°	200	26	8
B. proteus	½ ,, ,, ,, 20°	200	33	6

It is seen that the ratio of the germicidal activities of the two quinones varies (1) with the organism used from 4 to 17, (2) with the experimental conditions of time and temperature employed.

The *absolute* difference in the bactericidal power of the quinones is probably even greater than represented by the foregoing figures, because it has been found by experiments with viscose membranes that benzoquinone, although by far the more efficacious germicide, diffused somewhat more *slowly* than toluquinone.

The chemical action of quinones on proteins.

Equal amounts of the protein (0·2 g.) were allowed to react with solutions (20 cc.) of benzo- and tolu-quinone of the same concentration (0·2 %) under the same uniform conditions, and the amounts of the quinones reacting estimated by titration of the solutions at the end of a definite period. The results are expressed in Table III as ratios, representing amount of benzoquinone/amount of toluquinone reacting with the same amount of protein.

Table III.

Protein	Experimental conditions Temp. 18°	Ratio of reactivity
Edestin	½ hour	1·9
	1 ,,	2·2
	3 hours	1·5
*Wheat gliadin	½ hour	1·3
	1 ,,	0·9
	2 hours	0·6
	16 ,,	1·8
Glutenin	3 ,,	1·2
	24 ,,	1·5
Gelatin	72 ,,	1·8
	96 ,,	1·8

* Experiments with alcohol-soluble proteins are now of theoretical interest, as it has recently been found that such proteins occur as cell-contents of bacteria [Goadby, 1927].

In all these experiments the proteins were insoluble in water, and it was only necessary to pipette off the clear quinone solutions for estimations.

In the following experiments colloidal solutions of proteins were employed, viscose dialysers being used, so that the quinone solutions could be titrated at the end of the experiment in the absence of the protein phase. The following results (Table IV) were obtained.

Table IV.

Protein used	Period of experiment	Ratio of reactivity
Gelatin	24 hours	2·7
Edestin (in NaCl solution)	24 „	1·3
Albumin	1 hour	2·2
„	24 hours	1·0–2·4*

* From concentrations of quinones varying from 0·01–0·20 %.

The ratio of the chemical reactivity of benzoquinone to that of toluquinone may thus be slightly higher than in the case of the insoluble proteins, but in no case does the ratio attain the order observed in the bactericidal tests.

The action of the quinones has also been studied comparatively on yeast and *Bacillus xylinus*, the latter being grown on a large scale in glycerol-yeast-water. Weighed amounts of the organisms were placed in viscose dialysers, surrounded by the quinone solutions, as in the previous experiments.

Table V.

Organisms used		Ratio of reactivity of the quinones
1. Yeast	24 hours	1·0
	48 „	1·0
2. Dried yeast	24 „	1·5
	48 „	1·3
3. *B. xylinus*	24 „	1·1

In all three cases there is thus very little difference in the apparent activity of benzo- and tolu-quinone.

One possible explanation of the low ratios uniformly obtained with proteins and even with micro-organisms is that in addition to chemical action the quinones are *adsorbed* by proteins and other cell-colloids, with the result that the great difference between the reactivity of the quinones indicated by their chemical properties and bactericidal power is obscured. The relative amounts of benzo- and tolu-quinone adsorbed by animal charcoal were therefore next determined.

Table VI.

Final concn. of benzoquinone in water phase %	Amnt of benzoquinone adsorbed by 1 g. charcoal g.	Final concn. of toluquinone in water phase %	Amnt of toluquinone adsorbed by 1 g. charcoal g.
0·140	0·0088	0·176	0·0149
0·068	0·0069	0·082	0·0077
0·055	0·0055	0·037	0·0043
0·019	0·0022	0·015	0·0021
0·009	0·0019	0·010	0·0006

1 g. of animal charcoal was introduced into each of a series of solutions of the two quinones, varying from 0·01 to 0·20 %, adsorption being allowed to continue for 30 minutes. The clear solutions were then pipetted off free from charcoal and titrated. The results are tabulated in Table VI.

At higher concentrations toluquinone is adsorbed somewhat more freely than benzoquinone, but at low concentrations the reverse is the case. The amounts of quinone adsorbed increase with rise in concentration, but not proportionately, a marked falling off in the uptake being observed in the stronger solutions, as is frequently observed in cases of adsorption. When, however, the logarithms of the final concentration and of the amounts adsorbed are plotted against each other, a straight line results. Adsorption is thus evidently a factor in obscuring the greater chemical reactivity of benzoquinone in the case of such colloids as proteins. Toluquinone may be adsorbed to a greater extent than benzoquinone, although only a relatively small amount probably enters into chemical action.

Whether adsorption could be accepted as the principal factor in obscuring the relative chemical reactivity of the quinones could not be decided without further investigations, and experiments were therefore next carried out to compare the action of benzo- and tolu-quinone on the structural units of the proteins, *e.g.* the amino-acids and related substances.

Usually 0·2 g. of the substance (in solution or in suspension according to solubility) was allowed to react with 25 cc. of equimolecular solutions of the two quinones for definite periods, *e.g.* 0·5–48 hours, the solutions then being titrated and the amount of quinone entering into chemical action thus determined. The following results were obtained for the ratio: amount of benzoquinone/amount of toluquinone reacting.

Table VII.

Glycine	1·9–4·9
Alanine	3·9–6·2
r-Leucine	2·7
l-Leucine	0·6–2·6
Tyrosine: solid	0·7–2·1
Tyrosine: dissolved in *N*/10 HCl	5·8
Phenylalanine	6·0
Glutamic acid	4·3
Asparagine	1·7–6·2
Cystine: solid	1·8
Cystine: in solution	2·4
Mixture of diamino-acids ...	1·9–7·0
Tryptophan	1·5–3·0
Creatine	0·5–2·4
Creatinine	1·0
Glycylglycine	1·1
Glycine ester-HCl	2·8
Witte's peptone	1·4
Autolysed yeast	1·4–4·6
Sulphanilic acid	8

(Nucleic acid, glucosamine, and amides did not react with aqueous solutions of quinones in a period of 24 hours.)

A consideration of the foregoing observations shows that the highest ratios are obtained with such amino-acids as glycine, alanine, phenylalanine, tyrosine, and diamino-acids, and the marked superiority of benzoquinone as a germicide over toluquinone has a measure of correspondence in the greater readiness with which it reacts with such amino-acids.

The germicidal ratio of benzo- and tolu-quinone varies from 4 to 17 according to experimental conditions, whilst the ratio of their reaction-velocities with certain amino-acids may rise as high as 7, somewhat lower figures of 4–5 being frequently observed. Proteins, on the other hand, give much lower ratios of 1–2, but this is also found to be the case with certain amino-acids, *e.g.* cystine, tryptophan, and also in the case of peptone.

The low ratios obtained with proteins are therefore not entirely due to the complication of adsorption, but must have a structural explanation, and this view is supported by the fact that similarly low ratios are obtained with a protein structural unit, such as the peptide-grouping, *e.g.* glycylglycine, and also with the peptones. Glycine anhydride or diketopiperazine—apparently also a structural component of proteins—furthermore does not react with quinones.

The results suggest that the bactericidal action of the quinones is associated especially with their chemical reactivity towards comparatively simple cell-constituents, and not necessarily with the more complex colloidal substances such as the proteins. Furthermore, such simple amino-acids as alanine seem to be more fundamentally concerned than tryptophan.

The reactivity of the quinones towards amino-acids and proteins has also been studied in alcoholic solution, in view of the fact that the absorption spectra of the quinones are well known to be affected by organic solvents.

The following benzoquinone/toluquinone ratios were observed with glycine in 25 % alcohol.

Table VIII. *Ratios.*

	In 25 % alcohol
½ hour	14·9
1 „	12·6
2 hours	4·5
5 „	3·4
24 „	1·9

From these observations the interesting result emerges that alcohol very considerably increases the ratio of chemical reactivity of benzoquinone to that of toluquinone in the periods of ½–1 hour, but the ratio falls rapidly with the progress of the experiment. In the preliminary stages of the reaction benzoquinone is thus strongly activated by the solvent. The ratios of 12–15 are similar in magnitude to the extreme germicidal ratios occasionally observed, and the results suggest that there may be a cell-constituent which possesses the capacity of activating benzoquinone, thus increasing its efficacy to a greater extent than that of toluquinone. The presence of inorganic substances, *e.g.* the constituents

of Ringer's solution, slightly raises the quinone-ratio with glycine, but not sufficiently to be of significance in this matter.

In connection with these results it was of interest to study the influence of alcohol and acetone in dilutions of 25 % on the germicidal action of the quinones. The following results were thus obtained.

Table IX. *Germicidal concentrations.*

B. coli. 30 minutes at 20°.

	In water	In 25 % alcohol	In 25 % acetone
Phenol	1 in 115	1 in 300	1 in 140
Benzoquinone	1 in 35,000	1 in 400,000	1 in 125,000
Toluquinone	1 in 7,000	1 in 27,500	1 in 17,500
Xyloquinone	Insoluble	1 in 8,500	1 in 6,000
Thymoquinone	"	1 in 6,000	1 in 4,000
m-Dichloroquinone	1 in 45,000	1 in 300,000	—

It is seen that whilst the germicidal power of phenol is only increased approximately three times in the presence of alcohol, the action of the quinones is increased to a much greater extent, the efficacy of benzoquinone especially being enhanced, so that it is now germicidal in concentrations of 1 in 400,000. The results with acetone are similar, but less marked.

Dichloroquinone is exceptional to the general rule that the substituted quinones are less active than benzoquinone. It is, in fact, slightly more germicidal than the parent quinone, and also exhibits a very marked accession in bactericidal power in presence of alcohol.

It was subsequently found that this halogenated quinone reacts as readily as benzoquinone with glycine, and more readily than toluquinone. The halogen atom is thus favourable to high germicidal power and chemical reactivity.

Experiments were also put in hand to ascertain the influence of alcohol on the uptake of the quinones by proteins. Alcohol is known to diminish the adsorption of dissolved substances by colloids, and it was hoped therefore that it would be possible to exclude the interfering factor of adsorption and thus reveal the true relative reactivity of benzo- and tolu-quinone with proteins.

Weighed amounts of gelatin were allowed to react with solutions of varying concentrations of the two quinones (1) in water, and (2) in absolute alcohol for a period of 4 weeks in the dark at room temperature. A long period was allowed, because preliminary experiments showed that proteins continued to take up benzoquinone for at least 3 months. 1 g. of gelatin at the end of this period had combined with or adsorbed as much as 1·55 g. of benzoquinone.

The tabulated results (Table X) show that in aqueous solution benzoquinone is consistently taken up about twice as readily as toluquinone by gelatin. In alcohol, this is also true at low concentration, but at higher concentrations toluquinone tends to be taken up to a somewhat greater extent than benzoquinone. Furthermore, in aqueous solution the amount of benzoquinone adsorbed by the protein steadily rises with increasing concentration, whilst with toluquinone there is a tendency for the uptake to reach a maximum. In alcoholic solution, however, the uptake of both quinones reaches a limit.

Table X.

In water		In alcohol	
Initial concn. in water phase	Amnt of quinone taken up by 1 g. gelatin	Initial concn. in alcohol phase	Amnt of quinone taken up by 1 g. gelatin
Benzoquinone %		Benzoquinone %	
0·292	0·0892 g.	1·880	0·0220 g.
0·166	0·0425	1·535	0·0139
0·140	0·0329	1·050	0·0147
0·065	0·0219	0·554	0·0129
0·024	0·0111	0·339	0·0162
—	—	0·139	0·0077
Toluquinone %		Toluquinone %	
0·173	0·0268	3·066	0·0213
0·134	0·0233	1·006	0·0213
0·082	0·0166	0·507	0·0162
0·039	0·0061	0·289	0·0071
0·030	0·0045	0·071	0·0006

The interpretation of the results is difficult, and subsequently it occurred to the authors that the separation of the factors of adsorption and chemical action might be better attempted by studying the action of the quinones on desamino-proteins, in which the reacting NH_2 and NH groups have either been removed or substituted by nitroso-groups.

Finely divided egg-albumin was treated with nitrous acid by the method of Nakashima [1925] which effects complete deaminisation of the protein. Kjeldahl estimation showed that the product contained 13·8 % of nitrogen, as compared with 12 % in the original protein, showing a slight fixation of nitrogen, probably as nitroso-groups. The desamino-albumin failed to give a coloration with quinones. The uptake of benzoquinone and toluquinone by this product was then determined by the method already described.

Table XI.

$$\text{Ratio: } \frac{\text{Amount of benzoquinone taken up by 1 g. protein}}{\text{Amount of toluquinone similarly taken up}}$$

Initial quinone concentration varying from 0·01 to 0·15 %.

	Desamino-albumin	Albumin (control)
½ hour	0·54	—
24 hours	0·71–1·0	1·0–1·4

The ratio is now seen to vary from 0·5 to 1·0, toluquinone thus being taken up to a somewhat greater extent than benzoquinone by desamino-albumin; whilst with albumin itself the reverse is the case, the ratio varying from 1 to 1·4.

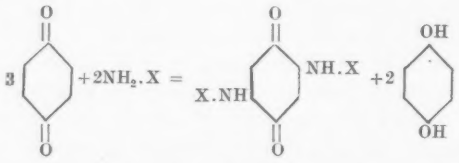
The results with the desamino-product are similar to those obtained with animal charcoal, which at certain concentrations adsorbed toluquinone more freely than benzoquinone.

The suppression of the chemical factor in the case of albumin by deaminisation thus results in a lowering of the quinone-ratio from on the average 1·2 to about 0·8. This change, however, is insufficient to account for the low combination ratios of the quinones with proteins. The results do not warrant the conclusion that proteins would yield a quinone-ratio of a similar numerical value

to that obtained with amino-acids or in the germicidal tests, if the chemical action were not masked by the physical process of adsorption. Adsorption must therefore be considered a secondary factor, and the low quinone-ratios observed with proteins must be due to an important extent to structural reasons, viz. the presence of (1) certain amino-acid radicles, of (2) the peptide structure, and (3) the diketopiperazine structure, as has been indicated earlier in this paper.

The conclusion is therefore drawn that the simpler cell-constituents, such as certain of the amino-acids, are mainly concerned in the mechanism of disinfection by the quinones.

According to Fischer and Schrader [1910] the general reaction is of the following type:



Three quinone molecules are thus utilised for every two nitrogen atoms.

Since 1 g. of gelatin used up 1·55 g. of benzoquinone, on the above equation the percentage of nitrogen in gelatin would be 13·6 %. Experimental determination showed the sample to contain 14·8 %. However, it is unlikely that every nitrogen atom present in the protein would enter into the reaction, and the result rather points to the conclusion that of the 1·55 g. of benzoquinone, an appreciable amount is adsorbed, and not chemically combined.

Summary.

1. Quinones readily undergo decomposition in aqueous solution in the presence of light, and the change is accompanied by a fall in bactericidal power.

2. Benzoquinone is from 4 to 17 times more active as a germicide than toluquinone, the exact ratio depending on experimental conditions. Notwithstanding, toluquinone is somewhat more diffusible through a membrane and more readily adsorbed by colloids than benzoquinone.

3. Alcohol increases the germicidal power of benzoquinone to a much greater extent than that of phenol and toluquinone. The interaction of benzoquinone and glycine is also greatly accelerated in the initial stages by alcohol.

4. Whilst there is relatively only a small difference in the *apparent* reactivity of the quinones towards yeast, bacteria, proteins, peptones, peptides, and even certain amino-acids, benzoquinone reacts up to 7 times as readily as toluquinone with other amino-acids.

5. Toluquinone is adsorbed to a somewhat greater extent than benzoquinone by charcoal and desamino-albumin. The difference, however, is not sufficiently great to justify the conclusion that adsorption is the primary factor in the protein experiments, and the evidence points to the conclusion that the

small differences in the uptake of benzoquinone and toluquinone by proteins have a structural explanation.

6. The germicidal power of the quinones is not necessarily due to their chemical interaction with the cell-proteins, but is to an important degree associated with their reactivity towards the simpler cell-constituents, such as certain of the amino-acids.

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XLIII. SEED FATS OF THE *UMBELLIFERAE*.

I. *HERACLEUM SPHONDYLIUM* AND *ANGELICA SYLVESTRIS*.

By THOMAS PERCY HILDITCH and EVELINE ELLEN JONES.

From the Department of Industrial Chemistry, University of Liverpool.

(*Received January 24th, 1928.*)

In 1909 Vongerichten and Kohler [1909] established the presence of an isomeric solid oleic acid, petroselinic acid, in the fatty oil of parsley seeds (*Petroselinum sativum*). In a recent analysis of the mixed fatty acids of this oil [1927] we found 76 % of petroselinic ($\Delta^{6:7}$-octadecenoic), 15 % of oleic, 5·5 % of linoleic, 2·5 % of palmitic and 1 % of higher saturated acids.

Scherer [1909] observed a solid oleic acid (presumably petroselinic acid) in the seed fats of two other Umbellates, *Pimpinella anisum* and *Foeniculum capillaceum*, and Palazzo and Tamburello [1914] have stated that the same acid is present in the fatty oil of *Hedera helix* seeds (Araliaceae). We have commenced the examination of a number of umbelliferous seed fats in order to ascertain whether this acid is invariably present therein, and how its relative proportion compares with that of the co-existent oleic ($\Delta^{9:10}$-octadecenoic) acid.

In this communication we deal with two common wild species of the Order, the seeds of which were collected in the early autumn of 1926 in the Wirral peninsula, Cheshire. The fruits were collected in dry weather, only those umbels which shed their fruits when knocked being chosen. The collected fruits were dried for some weeks in thin layers which were frequently turned, and were then shaken first in a coarse sieve to remove the small ray stalks, and then in a very fine sieve to remove any adhering dust. Due care was taken, by comparison with specimens in the Hartley botanical laboratories of this University, to ensure the respective identity of the two species, namely, *Heracleum sphondylium* (Cow parsnip or Hogweed) and *Angelica sylvestris* (Wild angelica).

In both cases the fruits, like those of many Umbellates, are characterised by relatively large winged ribbed carpels (mericarps), which contain notable amounts of fat in the vittae or oil ducts; it was, of course, not feasible to separate the seed proper from the rest of the fruit and therefore the fats examined consist of a mixture of the seed and carpel oils.

The seeds of the *Umbelliferae*, again, contain in general an unusually large proportion of non-fatty matter of various kinds which is extracted under the same conditions as the fat, and this adds to the complexity of the investigation.

In the case of *Heracleum* the non-fatty compounds present are mainly esters of lower fatty acids and alcohols, *e.g.* octyl acetate [Zincke, 1869; Möslinger, 1877], whilst *Angelica* seeds contain esters of valeric and other acids [Müller, 1881]. Precautions were taken to exclude these compounds as far as possible before the fatty acid mixtures were examined in detail.

The dried seeds were ground and extracted in a Soxhlet apparatus with ether; the recovered oil was in each case dark coloured, possessed a strong odour of lower fatty acids, and contained an appreciable amount of resinous matter. The yields and characteristics of the products obtained were as follows:

	Heracleum sphondylium	*Angelica sylvestris*
Dry seed taken (g.)	316	209
Crude oil (g.)	38·2	36·1
Crude oil %	12·1	17·3
Saponification equivalent	286·2	288·5
Free acid (as oleic) %	6·9	9·6
Iodine value	89·1	114·8
Non-saponifiable matter %	4·6	5·9

The whole of the fat was saponified and the soap extracted in dilute aqueous solution with ether in order to remove as much non-fatty material as possible; the fatty acids liberated from the soap were somewhat contaminated with resinified matter and also contained lower fatty acids, which were removed as far as possible by evaporation.

The crude mixtures of fatty acids were then treated with lead acetate in alcoholic solution in order to separate petroselinic (and any saturated) acid from ordinary oleic and linoleic acids. The lead salts deposited from the cold alcohol solution were recrystallised from a little ether, and in this way the following three groups of fatty acids were obtained from each of the two original fatty acid mixtures:

	Heracleum sphondylium			*Angelica sylvestris*		
Acids	g.	Iodine value	Mean equiv.	g.	Iodine value	Mean equiv.
Solids (*S*), from insoluble lead salts	6·0	66·7	281	6·3	76·1	284
Liquids (*E*), from lead salts insoluble in alcohol, but soluble in ether	8·1	107·4	275	4·7	118·0	274
Liquids (*A*), from lead salts soluble in alcohol	11·5	128·7	282	16·1	132·0	276
	25·6			27·1		

The acids from the soluble lead salt fractions deposited, on standing, small quantities of a crystalline acid, which was separated and not taken into account in the above lists. It was not obtained in sufficient amount for adequate investigation, but it does not appear to belong to the higher fatty acids; the substance has been encountered again in the similar fractions of acids from the fatty oil of cultivated parsnip, and we hope to make a more detailed examination of it during the work on this fat which is at present in progress.

It will be observed that the mean equivalents of all the groups of fatty acids are fairly close to those of acids of the oleic series; the acids (*E*) and (*A*) were in all cases dark coloured and somewhat contaminated with non-fatty

material, and this is probably the reason for mean equivalents lower than 280 having been recorded in some cases. Unfortunately, the amount of material at our disposal did not permit us to prepare the corresponding methyl esters and purify the latter by fractional distillation. We were able, however, by direct study of the six groups, to establish the identity of several fatty acids which were present, and these results, together with the iodine values of each fraction, enable us to give an estimate of the composition of both the Hogweed and Angelica fatty acids.

Fatty acids from Heracleum sphondylium.

(i) 6·0 *g. of solid acids from insoluble lead salts.* A portion (2·5 g.) was converted into the potassium salt and oxidised in dilute ice-cold alkaline solution by potassium permanganate [Lapworth and Mottram, 1925]. The acids recovered from this treatment contained a very small quantity of saturated fatty acid, which melted at about 51°; myristic acid was not present (as shown by mixed M.P. determination) and it is assumed that the substance was impure palmitic acid, possibly containing some stearic acid. The main product, consisting of dihydroxystearic acids, was crystallised twice from ethyl acetate, when it melted sharply at 121°; this melting-point was unchanged by admixture with authentic 6 : 7-dihydroxystearic acid prepared from parsley-seed oil.

Another portion (2·1 g.) was dissolved in glacial acetic acid (20 cc.) and treated with 35 % hydrogen peroxide solution (5 cc.). The product, after two crystallisations from ethyl acetate, consisted of white crystals, M.P. 114–115°, this being unaltered by admixture with the corresponding isomeric form of 6 : 7-dihydroxystearic acid prepared from parsley-seed oil.

(ii) 8·1 *g. of liquid acids from lead salts separated in ether solution.* Oxidation of these acids (4 g.) by the method of Lapworth and Mottram furnished a mixture of dihydroxy- and tetrahydroxy-stearic acids, a small amount of tetrahydroxystearic acid which melted at 168° being isolated. The main product consisted of dihydroxystearic acids, the melting-point of which could not be raised above 109–114° (indef.) by crystallisation from ethyl acetate, chloroform or alcohol; apparently both 6 : 7- and 9 : 10-dihydroxystearic acids were present.

(iii) 11·5 *g. of liquid acids from alcohol-soluble lead salts.* Similar oxidation of these acids (4·4 g.) gave tetrahydroxystearic acid (M.P. 150–152°) soluble in hot water, tetrahydroxystearic acid (M.P. 173°) insoluble in hot water and crystallised from ethyl acetate, and a fraction, more soluble in ethyl acetate than the preceding, giving a product which melted at 113–114° on further recrystallisation (apparently a mixture of dihydroxy- and tetrahydroxy-stearic acids).

The acids present in the original mixture from Hogweed seeds were thus palmitic, stearic (?), petroselinic ($\Delta^{6:7}$-octadecenoic), oleic ($\Delta^{9:10}$-octadecenoic) and linoleic acids.

Fatty acids from Angelica sylvestris.

The corresponding groups of these acids were examined in the same manner as the above with the following results.

(i) 6·3 *g. of solid acids from insoluble lead salts.* Aqueous alkaline oxidation of the acids (2·7 g.) with permanganate yielded the 6 : 7-dihydroxystearic acid, M.P. 120–121° (unchanged by admixture with the corresponding acid from petroselinic acid of parsley), together with a very small quantity of palmitic acid, which was not obtained quite pure (M.P. 58–59·5°).

Hydrogen peroxide in acetic acid converted the same acids (3·3 g.) into the isomeric 6 : 7-dihydroxystearic acid which melted, after crystallising twice from ethyl acetate, at 112·5–114° (no depression when mixed with an authentic specimen derived from parsley-seed petroselinic acid).

(ii) 4·7 *g. of liquid acids from lead salts separated in ether solution.* Aqueous alkaline oxidation with permanganate gave a small amount of tetrahydroxystearic acid, which melted at 169°, and a mixture of dihydroxystearic acids which could not be further resolved by crystallisation, and melted indefinitely at 111–114°.

(iii) 16·1 *g. of liquid acids from alcohol-soluble lead salts.* The aqueous alkaline permanganate oxidation yielded a mixture of hydroxylated acids, including tetrahydroxystearic acid (M.P. 170–171°) and a product similar to that obtained from the corresponding Hogweed fraction, which melted at 111–114° (indef.) and was apparently a mixture of dihydroxy- and tetrahydroxy-stearic acids.

The same acids were thus identified in the *Angelica* as in the *Heracleum* fatty acids.

If we assume that the fatty acid mixtures consist essentially of palmitic, petroselinic, oleic and linoleic acids, it is possible to arrive at a partial estimate of their quantitative compositions; since, however, the results of oxidation indicated fairly clearly that some petroselinic acid had passed into the ether-soluble lead salt fractions (*E*), it must be borne in mind that the petroselinic acid figures stated (which are based only on the solid acids (*S*) which definitely yielded 6 : 7-dihydroxystearic acid) do not represent the total amount of this acid.

Fatty acids of Heracleum sphondylium.

			Acid			
Fraction	g.	Iodine val.	Palmitic	Petroselinic	Oleic	Linoleic
S	6·0	66·7	1·1	4·9	—	—
E	8·1	107·4	—	—	6·6	1·5
A	11·5	128·7	—	—	6·6	4·9
	25·6		1·1	4·9	13·2	6·4
	%		4·3	19·1	51·6	25·0

Fatty acids of Angelica sylvestris.

			Acid			
Fraction	g.	Iodine val.	Palmitic	Petroselinic	Oleic	Linoleic
S	6·3	76·1	1·0	5·3	—	—
E	4·7	118·0	—	—	3·3	1·4
A	16·1	132·0	—	—	8·6	7·5
	27·1		1·0	5·3	11·9	8·9
	%		3·7	19·6	43·9	32·8

Whilst, therefore, petroselinic ($\triangle^{6:7}$-octadecenoic) acid is present in considerable amount in the seed fats from both *Heracleum sphondylium* and *Angelica sylvestris*, the proportion of this acid is much less than in the case of *Petroselinum sativum* seed fat (76·5 %). Even if a substantial amount of the lead salt of the acid had passed into solution on treatment with ether (lead petroselinate is, as a matter of fact, only sparingly soluble in ether), the amount of petroselinic acid would not be increased to more than 30–40 % of the combined fatty acids.

If it be surmised that petroselinic acid is characteristic of Umbellate seed-fats proper, and that the mericarp fat does not, perhaps, contain the isomeric oleic acid, figures of the order obtained would rather be expected, since the fruits of *Heracleum* and *Angelica* are notable for large mericarps or winged carpels. A decision on this point must be deferred until the studies of parsnip, carrot, celery, chervil and other Umbelliferous seed fats now in progress are further advanced.

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XLIV. THE AEROBIC AND ANAEROBIC METABOLISM OF THE COMMON COCKROACH (*PERIPLANETA ORIENTALIS*). III.

By JOHN GILBERT DAVIS
and WILLIAM KERSHAW SLATER (*Beit Memorial Research Fellow*).

From the Department of Physiology and Biochemistry, University College, London.

(*Received January 24th, 1928.*)

Introduction.

The common cockroach (*Periplaneta orientalis*) has long been known to live for considerable periods in the complete absence of oxygen. It has further been shown [Davis and Slater, 1926] that during such periods of anaerobiosis the insects go into debt for oxygen in a manner closely resembling that observed in man during severe exercise.

As the cockroaches are perfectly quiescent during the period of anaerobiosis, the debt must be incurred not as the result of special muscular activity, but in maintaining the resting metabolism of the animal as a whole. It is to be expected therefore that the amount of oxygen debt in a given period of anaerobiosis will be equal to the oxygen used at rest in an equal time in air; such a relationship was found to exist.

The investigations of Meyerhof, Warburg and their co-workers on lactic acid formation in isolated tissues suggest the possibility that the energy required for the resting metabolism of the cockroach under anaerobic conditions is supplied by the breakdown of glycogen to lactic acid, with the subsequent removal of lactic acid on the readmission of air.

Any removal of lactic acid during recovery will result in the adjustment of the acid-base equilibrium in the tissues with a corresponding retention of carbon dioxide. In experiments which have been made on the respiratory exchanges during the recovery period [Slater, 1927] there appears a marked drop in the respiratory quotient; the carbon dioxide evolved being lower than under normal conditions in spite of the increased oxygen intake. If, however, the observed carbon dioxide retention is compared with that calculated from the oxygen debt—assuming the relationship between lactic acid burnt and lactic acid removed which has been obtained for vertebrate muscle, viz. 1 : 4·4 [Furasawa and Hartree, 1926]—it is found in most cases to amount to less than half.

The present communication deals with the determination of the lactic acid content of the insects before and after anaerobiosis, in an attempt to establish directly the relationship between lactic acid and oxygen debt, which has been foreshadowed by the study of the respiratory exchanges.

Duration of life under anaerobic conditions.

In the earlier experiments considerable variations were observed owing to small traces of oxygen in the nitrogen, which was then in use as the inert gas. It was subsequently found that the insects can utilise oxygen at very low partial pressures; thus nitrogen containing 1 % of oxygen was not sufficient to produce the condition resembling anaesthesia, which characterises the beginning of the anaerobic state.

More recent experiments carried out with carefully purified hydrogen yielded better results; at 18° the insects suffered permanent damage if they were deprived of oxygen for more than two hours, the same condition being reached at 25° in one hour. It will be seen from the subsequent determinations that the concentration of lactic acid is approximately the same in each case. The average length of life without air is therefore shorter than was at first believed, but individual cockroaches differ widely; after four hours at 18° it is sometimes possible to obtain complete recovery, although this is comparatively rare.

The only obvious symptoms arising from prolonged oxygen lack are a swelling of the gut due to the accumulation of gases, and an increase in the brittleness of the insects, the legs coming away from the body at the slightest touch.

Method used for the estimation of lactic acid.

The estimation of lactic acid in small animals protected by a heavy chitinous cuticle presents special difficulties. Such animals have a very small weight relative to their body size, and only by selecting at least ten specimens can a sufficient weight of tissue be obtained. Though the use of a number of insects for each experiment reduces the error due to individual variations, it greatly increases the difficulties connected with crushing and extraction. The extraction is further complicated by the difficulty of breaking up the cuticle in order to allow the extracting fluid to reach the tissues.

The cockroaches were caught in the building and used as soon as possible, in order to avoid errors due to any metabolic changes resulting from captivity. The selected insects were weighed and introduced rapidly into a short length of glass tubing about 2·5 cm. in diameter, closed at each end with a tightly fitting stopper. The tube was immersed in a thermostat at the required temperature, and a very rapid stream of hydrogen passed. The hydrogen was prepared in a Kipp's apparatus, washed by solutions of potassium hydroxide, potassium permanganate and silver nitrate, and finally passed through a glass spiral in the thermostat in order to raise the temperature to that required for the experiment.

If the stream of hydrogen was sufficiently rapid, and the wash bottles had been previously filled with hydrogen, only a slight struggle took place, the cockroaches becoming motionless within two minutes. After five minutes the stream of gas was slowed down to 15 to 20 bubbles per minute and maintained at that rate throughout the experiment.

In the preliminary experiments wide variations in the lactic acid content were obtained; these variations were far greater after the periods of oxygen lack than in the control experiments. Wigglesworth [1927] has shown that the gut of the cockroach develops considerable acidity due to the presence of yeasts and bacteria, and that the chief product is lactic acid. In the later experiments, therefore, the gut was removed before the lactic acid was estimated. The general results after removal of the gut were much lower, and the variations after anaerobiosis fell within the limits of error to be expected in this estimation.

The insects to be examined were broken up in a 4 % solution of trichloroacetic acid, and the mash was allowed to stand. The clear filtrate from this was freed from sugar by the copper-lime method, and the lactic acid in the sugar-free solution estimated by Meyerhof's modification of the Clausen method. Duplicate determinations were made in each case, and the results rejected unless they were in reasonable agreement.

It was necessary before proceeding to the actual experimental work to ascertain the extent to which the tissue was being extracted. A number of estimations were made on whole cockroaches, using different methods of disintegration, the results of which are given below. It will be seen that mincing with a vegetable mincer is by far the most efficient method. This method was adopted throughout the later experiments.

Table I.

Method	Number of determinations	Mean of lactic acid found mg. %	% mean deviation
Grinding alone	3	8·5	5·9
Grinding with sand	6	28·8	10·4
Mincing with a vegetable mincer	5	40·9	7·1

There remained now only the length of time of extraction as a further possible source of error. Experiments were made in which the mash was allowed to stand for various times, and it was found that after 1 hour no further extraction occurred. This time was therefore fixed as the minimum time for extraction.

The cockroaches were selected as far as possible to weigh between 0·3 and 0·4 g. Sex was found to have no marked influence on the lactic acid content; it was therefore unnecessary to take any special precautions in this connection. In actual practice it was found convenient to use a preponderating number of females.

Experimental results.

Table II.

Series and temp.	Duration of anaerobiosis	Number of results	Lactic acid found mg. %	% mean deviation	Excess lactic acid: Found mg. %	Excess lactic acid: Calc. mg. %	Ratio found / calc.
I	Control	4	18·8	20	—	—	—
II 14°	1 hour	2	37·9	6	19·1	93·0	0·21
III 14°	2 hours	4	55·0	1·7	36·2	186·0	0·20
IV 18°	10 minutes	2	27·5	5·4	8·7	23·0	0·37
V 18°	1 hour	5	57·2	7·4	38·4	138·0	0·28
VI 18°	2 hours	3	80·6	2·3	67·8	276·0	0·25
VII 25°	1 hour	3	72·6	4·6	53·8	270·0	0·20
VIII 25°	2 hours	3	98·8	10·0	80·0	540·0	0·15

In Table II are summarised the results of the lactic acid determinations made immediately after a period of anaerobiosis. Experiments were made at three different temperatures, viz. 14°, 18°, and 25°. It was necessary to obtain a basal value for the lactic acid content of the cockroach after removal of the gut. In order to do so the cockroaches were lightly chloroformed and the guts

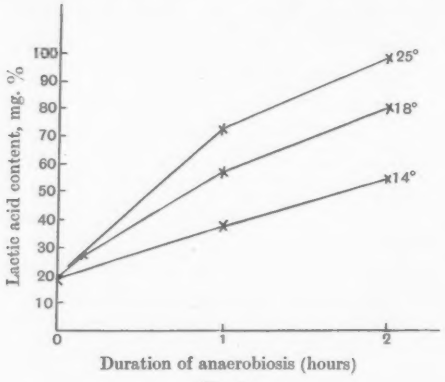

Fig. 1.

removed, the rest of the estimation following the normal method. The results of these determinations are given in the first series. Owing to the smallness of the value the mean percentage deviation is high. The figures given in Series IV were obtained in order to show that the slight struggle consequent on the admission of the hydrogen is responsible for no great production of lactic acid. It is difficult to control experiments over so short a period, and it would be unwise to place any absolute reliance on these figures, but they indicate quite clearly that the onset of anaerobiosis is accompanied by little or no lactic acid formation.

The remaining experimental figures require no special explanation. They show quite clearly that the concentration of lactic acid increases with the period of anaerobiosis, and also with the temperature at which the experiments are

carried out. Fig. 1 shows these relations graphically. The relation between the concentration of lactic acid and the period of anaerobiosis is for the lower values of lactic acid approximately linear. This relationship does not appear to hold for the higher values, there being a noticeable drop in output during the second hour at 25°, corresponding to the approach of the limit of anaerobic life.

It was now necessary to show that the lactic acid disappeared during recovery. As the result of experiments described in a previous communication [Davis and Slater, 1926] it was found that after 1 hour of anaerobic life at 25° a period of 3 hours was required before the whole of the oxygen debt had been recovered. A batch of cockroaches was exposed to hydrogen for 1 hour at 25°, and analyses made of a number of insects immediately after the admission of air, and at intervals of 1, 2 and 3 hours. The estimations were carried out in duplicate and the results are shown in Fig. 2, together with the graph of the oxygen debt recovery for purposes of comparison. It will be seen that the curves correspond within the limits of experimental error, the animals having returned to normal at the end of 3 hours.

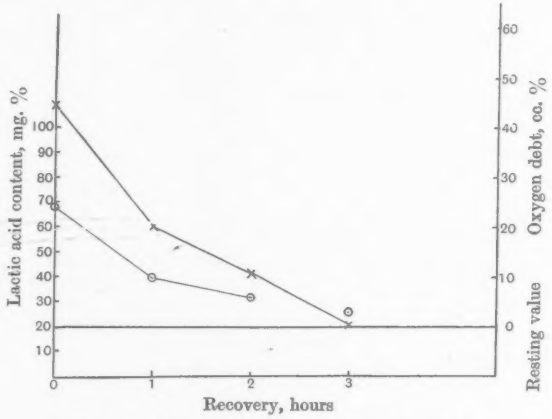

Fig. 2. × Oxygen debt ⊙ Lactic acid content

Discussion.

The formation of lactic acid during anaerobiosis, and its removal during recovery, is in accordance with the original assumption that the energy required for the maintenance of life in the absence of oxygen is provided by the breakdown of glycogen to lactic acid. It is possible to check this hypothesis further by comparing the quantity of lactic acid found with that calculated from the respiratory exchanges.

If we assume that the body tissues of the cockroach behave in the same way as vertebrate tissues, so that for each molecule of lactic acid, or its carbohydrate equivalent, burnt, 4·4 molecules are removed, we can calculate the

amount of lactic acid which should be associated with a given oxygen debt. Further, the oxygen debt is known to be equal to the oxygen which would have been used during the period of anaerobiosis if the animals had remained at rest in air. The oxygen intake at rest for 25° has been measured, and for cockroaches of the type used the average figure is 450 mm.^{3} per g. per hour. Vernon [1897] has measured the carbon dioxide output at lower temperatures, and from his figures it is possible, taking the respiratory quotient of the freshly caught cockroach at 0·9, to calculate the oxygen debt for 14° and 18°.

The lactic acid values calculated in this way are given in Table II, together with the ratio lactic acid found/lactic acid calculated. It is quite clear from these figures that the assumptions do not hold quantitatively, yet the uniformity of the ratio suggests that the general thesis is in part correct.

The low lactic acid content of the cockroaches may be explained by assuming the oxidation of a larger proportion of the lactic acid; in fact the figures suggest its total oxidation with no accompanying recovery. In Table III are shown the values for lactic acid found compared with those calculated for complete oxidation.

Table III.

Series	Excess lactic acid found mg. %	Excess lactic acid calc. for complete oxidation mg. %	Ratio found / calc.
II	19·1	20·7	0·92
III	36·2	42·2	0·86
IV	8·7	5·2	1·67
V	38·4	31·3	1·23
VI	67·8	62·5	1·08
VII	53·8	61·6	0·88
VIII	80·0	122·0	0·66
			Av. 1·04

Except in abnormal cases of Series IV and VIII, the ratio of the lactic acid found to that calculated lies near to unity.

Support is given to this apparent absence of any recovery process by the abnormally high carbon dioxide output of the cockroach when compared with other animals. Thus, according to Vernon [1897], it is five times as great as that of the frog, and eight times as great as that of the earthworm, at 20°.

It is possible to suggest other explanations, but they do not fit so closely to the observed facts as the assumption that the whole of the lactic acid is burnt, without any accompanying recovery. For example, an extension and adaptation of the suggestion put forward by Gerrard [1927] in the case of isolated nerve, that an oxygen carrier is present in the tissue, would partly explain the facts, but it would not account for the linear relationship between lactic acid production and the period of anaerobiosis, or for the close agreement in time between the oxygen debt recovery and the disappearance of lactic acid. Moreover, the carrier would have to be present in quantities sufficient to deal with 240 mg. % of lactic acid.

Again, a second process at present unknown may occur simultaneously with the lactic acid formation. Without a complete analysis of the cockroaches it is not possible to set aside this explanation, although it is difficult to reconcile it with the close relationship between lactic acid production and oxygen debt.

Until further evidence is available it seems reasonable therefore to accept the complete oxidation of the lactic acid as a working hypothesis.

Summary.

(1) The lactic acid content of cockroaches (*Periplaneta orientalis*) has been determined after various periods of anaerobiosis and during the recovery process.

(2) Lactic acid accumulates during anaerobiosis and returns to the resting value slowly during the recovery process.

(3) The excess lactic acid amounts to only about one-fifth of that calculated from the oxygen debt on the assumption that the recovery takes place as in vertebrate muscle.

(4) If, on the other hand, it is assumed that all the lactic acid is burnt without recovery, then the excess lactic acid agrees within the limits of experimental error with that calculated from the oxygen debt.

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XLV. THE ANAEROBIC METABOLISM OF THE EARTHWORM (*LUMBRICUS TERRESTRIS*).

By JOHN GILBERT DAVIS
AND WILLIAM KERSHAW SLATER (*Beit Memorial Research Fellow*).

From the Department of Physiology and Biochemistry, University College, London.

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Introduction.

The investigation of the anaerobic metabolism of the earthworm was commenced simultaneously with that of the cockroach [Davis and Slater, 1926, 1928; Slater, 1927], and carried out in a similar manner.

Owing, however, to the greater variations which occurred in the experimental results obtained from the earthworm the investigation was for a time abandoned. It was recommenced in order to ascertain whether the apparently abnormal recovery process observed in the cockroach applied to other distantly related invertebrates.

In the case of the earthworm there is considerable evidence available as to the nature of the anaerobic metabolism [Lesser, 1907 *et seq.*]. Lesser, following the methods used by Weinland [1901, 1902] in the investigation of intestinal worms, obtained results from which he concluded that the energy for anaerobic life is supplied by the breakdown of glycogen to carbon dioxide, a fatty acid (probably valeric), and an unidentified product. More recent work has shown that Weinland was in error in the conclusions which he had drawn [Fischer, 1924; Slater, 1925], and hence doubt was also thrown on the analogous deductions made by Lesser in the case of the earthworm. It will be shown later that Lesser's results may be interpreted in support of the general view that the source of anaerobic energy is due to the breakdown of glycogen to lactic acid.

The methods employed in the investigation of the earthworm are almost identical with those used in the case of the cockroach, and in consequence details are given only when variations occur. Full descriptions will be found in the publications referred to above.

Method.

The determinations which have been made consist of the measurement of the oxygen intake at rest under normal conditions and following upon a period of anaerobiosis and the estimation of the lactic acid content before and after anaerobiosis.

In determining the oxygen intake certain factors had to be considered which did not present themselves in the case of the cockroach. It was necessary to decide whether the worms should be used with earth in the gut, or whether they should be kept under such conditions that before the experiment they would empty their guts completely. There were obvious objections to each procedure. Thus, if the guts contained earth, the weight of the earth could not be taken into account, and throughout the experiment a steady contractile movement of the gut wall occurred. On the other hand, if the guts were emptied before the experiment the animals appeared to behave abnormally, with varying activity of the gut muscle. After careful consideration it was decided that when the average of a number of determinations made under similar conditions was taken, the lesser error would occur when the normal worms with full guts were used.

The second difficulty arose from the sensitivity of the worms to direct light. It was found at an early stage of the work that direct light from the thermostat lamps caused the oxygen intake to rise to approximately double the value obtained in dull diffused light. The rise in metabolic rate was unaccompanied by any visible irritation. All the results given below have been obtained in the absence of any direct illumination.

In determining the lactic acid content it was not possible as in the case of the cockroach to remove the gut, so the whole animals were minced and extracted with a 4 % solution of trichloroacetic acid. It should be noted in this connection that the presence of the gut in the cockroach leads to high values for the resting lactic acid, and to irregular values after anaerobiosis; the average of the latter values, however, exceeds the corresponding average for the gutted insects by no more than the difference between control experiments on the ungutted and gutted insects.

Experimental results.

Resting metabolism. The normal resting oxygen intake was determined in order to ascertain the influence of size and other factors on the metabolic rate. As in the case of the cockroach, the larger animals showed a definitely lower oxygen intake. If any marked movement other than that of the gut occurred during the experiment, the result was rejected. In Table I are given the results for nine worms in order of increasing weight.

Table I. *Oxygen intake of* Lumbricus terrestris *at* 25°.

Body weight g.	Resting oxygen intake mm.³/g./hour
0·26	200
0·33	183
0·90	80
1·13	107
1·29	88
1·35	95
1·40	100
1·92	42
1·93	66

Anaerobic metabolism. Purified hydrogen was used to remove the air in the respiration chamber, and a slow stream maintained throughout the period of anaerobiosis.

As in the case of the cockroach the animals went into debt for oxygen during the anaerobic period and used an excess of oxygen afterwards to refund this deficit. The agreement was not good owing to the many variable factors, but the results of five typical experiments given in Table II show the general relationship between the oxygen of which the animal has been deprived and the excess which is used during recovery.

Table II. *Recovery of* Lumbricus terrestris *after anaerobiosis. Temp.* 25°.

Exp.	Wt. of worms g.	No. of worms	Period of anaerobiosis	Oxygen debt mm.³	Recovery Excess oxygen used (mm.³) hours recovery ½	1	1½	2
1	4·19	3	1 hr. 0 min.	450	251	411	418	419
2	3·84	2	1 10	360	176	269	314	314
3	3·31	4	1 10	177	150	172	182	198
4	4·09	2	1 0	329	274	373	388	376
5	3·86	2	1 0	278	258	353	—	—

There is a marked difference between the time taken for recovery in the cockroach and in the earthworm. Whereas in the earthworm the recovery is complete in most cases within a time equal to that of the period of anaerobiosis, in the cockroach the period is three times as long.

Lactic acid content. Lactic acid was estimated in freshly caught earthworms, in order to establish a resting control, and also after 1 hour's anaerobiosis at 25°. The results are given in Table III together with the calculated lactic acid content on the basis of the relationship in vertebrate muscle, *i.e.* 1 part of lactic acid oxidised for 4·4 removed. The average resting oxygen intake was assumed for purposes of this calculation to be 120 mm.³/g./hour, as the worms used weighed between 0·5 and 0·8 g.

Table III. *Lactic acid content of earthworms.*

Number of experiments	Time of anaerobiosis	Temp.	Lactic acid found mg. %	Mean deviation %	Excess lactic acid over resting Found mg. %	Calc. mg. %	Ratio Found / Calc.
4	Control	—	41	9·0	—	—	—
5	1 hour	25°	122	12·5	81	72	1·1

Discussion.

From the experimental results it seems reasonable to conclude that the oxidation process is merely deferred during anaerobiosis until oxygen is re-admitted, when the products of metabolism are removed by an excess of oxygen equal to that which would have been used had the normal metabolism continued undisturbed. These results are compatible with the breakdown of glycogen to lactic acid followed by the oxidative removal of the latter.

The results obtained in the estimation of the lactic acid confirm this view, in that the anaerobic life is accompanied by a considerable accumulation of this acid.

The relation between the oxygen debt and the excess of lactic acid, unlike the corresponding relationship in the cockroach, shows a close agreement with the conditions found in vertebrate tissue, the oxygen used in recovery being only about one-fifth of that necessary for the complete oxidation of the accumulated acid. It must be concluded therefore that this complete oxidation, if that be the true explanation of the results obtained on the cockroach, does not extend to the annelid worms, and further investigation will be necessary in order to define the biological boundaries within which the synthetic recovery process is not found. The result in the case of the earthworm agrees with its low oxygen intake as compared with the cockroach, and it would seem reasonable to search for other examples of complete oxidation amongst those animals whose oxygen intake is relatively high.

It is necesssary to comment shortly on the earlier work of Lesser, and to try to explain the results he obtained in the light of the present investigation. Lesser's experimental findings were briefly as follows.

(1) During anaerobiosis glycogen is used in excess of the normal resting consumption.

(2) Carbon dioxide is evolved during anaerobiosis.

(3) The respiratory quotient is depressed during recovery.

(4) An excess of oxygen over the resting intake is used during recovery.

(5) Small quantities of a fatty acid are produced. This acid is volatile in steam and is probably valeric acid.

In explanation of these facts Lesser suggested that during anaerobiosis glycogen broke down to give carbon dioxide, the volatile fatty acid and another product which he failed to identify. Thus an excess of glycogen would be required during the anaerobic period, carbon dioxide would be evolved and the fatty acid would accumulate; following the re-admission of air, excess oxygen would be required to burn the fatty acid, and simultaneously the respiratory quotient would be lowered.

The facts, however, can be explained equally simply on the basis of the breakdown of glycogen to lactic acid, with the subsequent removal of the acid by oxidation and re-synthesis of glycogen. Thus, during the absence of oxygen, glycogen will be used without re-synthesis, and therefore, instead of the disappearance of only 1 part of each 4·4 parts degraded to lactic acid, the whole of the glycogen will be temporarily lost. The excess glycogen used during anaerobiosis will be 3·4 times as large as the normal consumption. If the value 100 mm.^{3}/g./hour is assumed for the normal oxygen intake, it is possible to calculate the resting glycogen consumption in air, and hence the excess glycogen required in the absence of oxygen. This calculated value is given in column 4 of Table IV, whilst the corresponding experimental values are given in column 5. The agreement on the whole is good.

The accumulation of lactic acid during anaerobiosis will change the acid base equilibrium of the tissues with a resultant evolution of carbon dioxide. A corresponding change in the opposite direction will occur during recovery as the lactic acid is removed. These changes can account for the evolution of carbon dioxide during anaerobiosis and the lowered respiratory quotient during recovery. In column 6 of Table IV are given values for the calculated output of carbon dioxide during anaerobiosis, on the assumption that the excess of glycogen disappearing, as found by Lesser, is turned into lactic acid and neutralised by sodium hydrogen carbonate; in column 7 are the corresponding values for carbon dioxide evolved during anaerobiosis, as found by Lesser. It will be noted that there is a fairly uniform relationship between the values calculated and found, and that the calculated in all cases except Exp. XLI is larger than the observed. This is to be expected, as bases other than carbonates must take part in the buffering.

The increased oxygen consumption requires no comment. The presence of volatile fatty acids in quantities less than one-tenth of the excess of glycogen used can only be explained by the activity of bacteria in the intestinal tract of the worm, a similar explanation to that suggested in the case of the intestinal worm.

Table IV. *Recalculation of the experimental data given by Lesser.*

Experiment number (Lesser)	Weight of worms g.	Period of anaero-biosis hours	Excess glycogen		Carbon dioxide evolved during anaerobiosis	
			Calc. mg.	Found mg.	Calc. mg.	Found mg.
XXXVII	62·54	4	112	169	84	47
XXXVIII	98·20	5	221	163	81	72
XXXIX	119·04	4	212	208	104	76
XLI	74·00	5	167	49	24	55
XLIX	99·90	6	270	333	165	53

It may be assumed, therefore, that the data given by Lesser are not incompatible with the present conception of the source of anaerobic energy in the earthworm, and in view of the experimental evidence given above it seems reasonable to accept the breakdown of glycogen to lactic acid as the metabolic process involved in the maintenance of anaerobic life.

Summary.

(1) The earthworm (*Lumbricus terrestris*) goes into debt for oxygen when it is kept under anaerobic conditions, using an excess volume of oxygen during recovery in air equal to that which it would have consumed during the anaerobic period.

(2) During the anaerobic period lactic acid accumulates in the tissues of the worm.

(3) The amount of lactic acid formed in a given time is related to the oxygen debt in the same way as in isolated vertebrate tissue. Thus in distinction to the cockroach there is a re-synthesis of glycogen.

(4) The data previously obtained by Lesser for the changes taking place in the earthworm during anaerobiosis can be explained by the breakdown of glycogen to lactic acid without the assumption of any special mechanism.

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XLVI. THE ALDEHYDE OXIDASE OF THE POTATO.

By FREDERICK BERNHEIM.

From the Biochemical Laboratory, Cambridge.

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The aldehyde oxidase of the potato was first studied by Bach [1913]. He showed that the potato reduces sodium nitrate to nitrite in the presence of acetaldehyde and that an enzyme is responsible. He stated, however, that the system potato plus aldehyde would not reduce methylene blue. In this respect it differed from the aldehyde oxidase of animal origin. Michlin [1927] was able to precipitate the enzyme from a watery extract of potato by acetone, and stated that the dried powder reduced nitrates but not methylene blue. It was therefore thought interesting to compare further the properties of the potato with the animal aldehydase, and in the course of the investigation it was found that preparations of the potato enzyme would under suitable conditions reduce both nitrate and methylene blue. It is thus possible to correlate the specificities of the plant and animal aldehydases.

Preparation of the enzyme.

Old and new potatoes have been used with similar results. When potatoes are minced, the juice squeezed out contains approximately half the enzyme. The other half seems to be firmly fixed to the potato pulp, and is not extracted by washing with large quantities of water, by grinding with ether and then extracting with water, or by dilute alkalis. Therefore, for the preparation of the enzyme the juice was used. Two pounds of potatoes were finely minced and 300 cc. of juice pressed out through linen. The cloudy liquid was saturated with ammonium sulphate and the precipitate filtered off with suction. The precipitate contains all the enzyme. It was then washed several times with saturated ammonium sulphate solution and finally dissolved in 150 cc. of distilled water. The solution contains a cloudy suspension which is filtered off. For the further purification the method used by Dixon and Kodama [1926] for the purification of the Schardinger enzyme was used. 100 cc. of the clear brown filtrate are treated four times with 3 g. of charcoal (Merck's medicinal). The resulting solution has lost none of its activity, but about 90 % of its protein, and is very light brown in colour. It is then made slightly acid and 5 g. of kaolin are added. All the enzyme is adsorbed and is eluted by 5 % sodium carbonate solution. It is neutralised with dilute acetic acid and

dialysed for 4 hours. The solution has about 5 % of its original protein and about one-third of its activity as the following experiment shows.

Exp. 1. The activity of the enzyme during the various stages of preparation was determined as follows: 1 cc. of the solution to be tested was put in a vacuum tube with 2 cc. buffer p_H 7·3, 1 cc. 5 % sodium nitrate and 0·5 cc. 1 % acetaldehyde. The tubes were thoroughly evacuated and kept at 37° for 2 hours. The nitrite formed was determined by adding 2 cc. of the Griess-Ilosvay reagent to each tube and comparing the colour resulting, after 15 minutes, in a colorimeter. The protein was estimated by taking 1 cc. of the solution to be tested and adding 5 cc. of a 2 % solution of sulphosalicylic acid, and after a given interval comparing in a nephelometer. Tube *A* contains the original juice, tube *B* the ammonium sulphate precipitate dissolved in water and filtered, tubes *C*, *D*, *E*, *F*, the solution of the precipitate after the first, second, third and fourth treatment with charcoal respectively, and tube *G* after treatment with kaolin.

Tube	Activity	Protein %
A	+ + +	100
B	+ + +	50
C	+ + +	32
D	+ + +	26·5
E	+ + +	18·3
F	+ + +	12·5
G	+	4·7

It is obvious that there is no advantage in the treatment with kaolin and unless otherwise specified the enzyme was used after the fourth treatment with charcoal in the following experiments.

The solution was then saturated with ammonium sulphate and the precipitate centrifuged off and dried in a vacuum desiccator. A grey powder was obtained which is readily soluble in water.

Specificity of the enzyme.

(*a*) *Hydrogen donators.* Using nitrate as the acceptor various substances beside aldehyde were tried as hydrogen donators, all of which were negative. They included the following: ethyl alcohol, acetone, diethyl ketone, glucose, sucrose, the sodium salts of lactic, citric, succinic, malic, tartaric, acetic, formic, and pyruvic acids, quinine, morphine, tyrosine, cysteine, catechol, hypoxanthine, and reduced glutathione. None of these reduce nitrate alone. All the aldehydes tried were distinctly positive except *p*-dimethylamino-benzaldehyde which, probably because of its insolubility, reduced but slightly. The figures in the following table represent the amount of nitrate reduced in arbitrary units, the reduction by acetaldehyde being taken as ten units.

Exp. 2. 1 cc. potato juice, 5 cc. buffer p_H 7·3 and 1 cc. 5 % sodium nitrate were placed in each of nine tubes with 0·05 cc. of the aldehyde to be tested. The tubes were then evacuated and incubated at 37° for 2 hours. 2 cc. of the Griess-Ilosvay reagent were then added and the resulting colour compared in a colorimeter.

Aldehyde added	Reduction of nitrate
Formaldehyde	2
Acetaldehyde	10
Propaldehyde	4
Benzaldehyde	9
Valeraldehyde	8
Anisaldehyde	9
Cinnamaldehyde	7
Citral	8
p-Dimethylaminobenzaldehyde	0·5

The differences in the amount of reduction are probably due to differences in toxicity and solubility.

(*b*) *Hydrogen acceptors.* It has been found that a variety of hydrogen acceptors can be substitued for nitrate in the system. Methylene blue, the Clark indicators (dyes of the indophenol and indigo groups with known oxidation-reduction potentials), quinone, and *m*-dinitrobenzene are reduced by the enzyme plus aldehyde but not by the enzyme alone. These results have been obtained a great number of times and with a variety of potatoes.

The reduction of methylene blue has been studied most extensively. The rate of reduction varies according to the preparation of the enzyme. In the untreated juice 1 cc. of 1 : 5000 methylene blue is reduced in about an hour; and the accelerating effect of aldehyde is comparatively small. The probable reason for this is that there are in the untreated juice substances which reduce methylene blue spontaneously, or others, being aldehydic in nature, which act as hydrogen donators. Consequently addition of aldehyde has little effect. The well-washed pulp, which contains about half the enzyme (as estimated from its nitrate reduction), on the other hand, reduces methylene blue slowly in the presence of aldehydes and much more slowly (negligibly for the purposes of the experiment) alone. This slow reduction in contrast to the free enzyme might be explained on the grounds of adsorption. The pulp adsorbs the dye in considerable quantities and may render it less available for the enzyme, thus increasing the reduction time. The ammonium sulphate preparation of the enzyme reduces rapidly in the presence of aldehyde and not at all alone. The following experiment shows the time relations of the various reductions using roughly equivalent amounts of enzyme.

Exp. 3. 2 cc. buffer p_H 7·3 and 1 cc. methylene blue 1 : 5000 were placed in each of six tubes. In tube *A* 2 cc. untreated potato juice were added and in tube *B* the same, plus 0·5 cc. 1 % acetaldehyde. In tube *C* was placed 0·4 g. washed potato pulp, and in tube *D* the same, plus 0·5 cc. acetaldehyde. In tube *E* 2 cc. purified juice were added, and in tube *F* the same, plus 0·5 cc. acetaldehyde. The reductions were carried out anaerobically at 37°.

Tube	Reduction time (mins.)
A	70
B	60
C	No reduction
D	480
E	No reduction
F	120

The reduction of the Clark dyes was carried out with the enzyme purified with charcoal and kaolin. The dyes were not reduced by the enzyme alone but were when aldehyde was added to the system. The time of reduction, however, was not obviously dependent on the r_H of the dye or on its chemical constitution. No. 5 which was reduced first was in the red form, but so was No. 7 which reduced several hours more slowly than No. 4, the last, like all the others, being in the blue form. Inasmuch as similar irregularities appear with a preparation of the aldehydase from liver, it must be concluded that the free energy of the reaction is not the factor determining the rate in these aldehyde systems. The determining factors are unknown. Boiled enzyme as control plus aldehyde does not reduce or at most very slowly in times far exceeding those of the enzyme aldehyde reduction. Finally *m*-dinitrobenzene which gives no oxidation potential is easily reduced. The following experiment gives the reduction times of the dyes.

Exp. 4. The p_H of the solutions was 7·3, and the experiments were carried out at 37°. The dyes, through the kindness of Dr Needham, were obtained from Dr Clark, and were made up in solutions equivalent to a 1 : 5000 methylene blue solution. 1 cc. enzyme purified with charcoal and kaolin, 2 cc. buffer, 1 cc. of the dye to be tested, and 0·5 cc. 1 % acetaldehyde were placed in each tube and the reduction time was determined. All the dyes when subsequently shaken in air were re-oxidised completely. The dyes are given by their numbers (for a list of names and numbers see Dixon [1926]). Number 3 *a* is methylene blue.

Dye	Reduction time with aldehyde	Reduction time without aldehyde
1	Slightly in 18 hrs.	No reduction
2	,, 18	,,
3	,, 18	,,
3 *a*	182 mins.	,,
4	140	,,
5	88	,,
6	660	,,
7	510	,,
8	540	,,
9	340	,,

Acetaldehyde was the only aldehyde studied systematically. Benzaldehyde was tried with methylene blue. The reduction time was a little longer than when acetaldehyde was used with the same amount of enzyme.

Oxygen may or may not act as a hydrogen acceptor. It was impossible to measure the oxygen uptake of the enzyme aldehyde system because it was found that aldehyde and oxygen rapidly destroy the enzyme. Two tubes, one containing 1 cc. of enzyme, 3 cc. buffer p_H 7·3, and 1 cc. water, the other the same except that 1 cc. 1 % acetaldehyde was substituted for the water, were allowed to stand open to the air for an hour at 37°. At the end of that time aldehyde was added to the tube containing none, methylene blue or nitrate added, and the tubes were evacuated. It was found that the incubation with aldehyde inhibited both reactions completely. On the other hand, incubation

with aldehyde in the absence of oxygen inhibits the activity of the enzyme comparatively slightly. Therefore, when the enzyme is shaken in a Barcroft apparatus with aldehyde, there is a very slight initial uptake and then a complete cessation. Nor does the addition of catalase to break up hydrogen peroxide that may be formed, and may be responsible for the inactivation of the enzyme, increase the uptake considerably.

In order to determine whether quinone is reduced, the reduction potential of the system was measured before and after adding aldehyde. The reduction potential of the purified juice alone shows a regular drift, the rate of which is unaffected by the addition of aldehyde. The enzyme plus quinone, however, gives a perfectly steady potential which when aldehyde is added shows a marked reduction. Enzyme boiled for 15 seconds plus aldehyde shows a reduction, but a much slower one, and enzyme thoroughly boiled for 5 minutes gives no reduction as the following curves show. 1·5 cc. of enzyme and 5 cc. of buffer p_H 7·3 were used. The experiments were carried out in nitrogen at 20°, and the potential measured with gold electrodes.

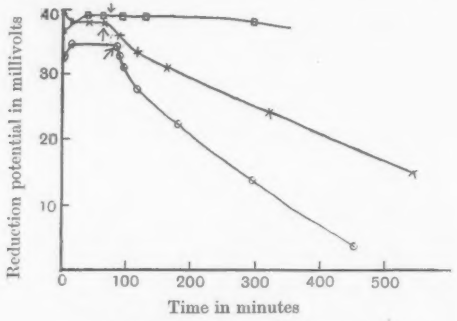

Fig. 1. Reduction of quinone.

□——□ Enzyme boiled 5 minutes. ×——× Enzyme boiled fifteen seconds.
○——○ Enzyme not boiled. → 1 cc. acetaldehyde added.

The effect of nitrate on the reduction of methylene blue was then tried. If the reductions of the dye and nitrate are due to the same system it would be expected that the two hydrogen acceptors would compete, that is, the methylene blue reduction would be retarded in the presence of nitrate. This was found to be the case, as the following experiment shows, and suggests that the same system is responsible for the nitrate and methylene blue reductions. If different systems are responsible it would be necessary to assume that the presence of nitrate is in some way toxic to the methylene blue-reducing system.

Exp. 4. Two tubes were set up; tube *A* containing 1 cc. of enzyme, 3 cc. buffer p_H 7·0, 1 cc. methylene blue, 0·5 cc. 1 % acetaldehyde, and 1 cc. water; tube *B* containing the same except that 1 cc. 5 % sodium nitrate was substituted for the water. The reductions were carried out at 37°.

ERRATUM

Vol. 22, p. 348, Fig. 1. The description of the ordinates should read "Observed E. M. F. against a saturated calomel electrode."

Tube	Reduction time (mins.)
A	90
B	115

It was shown by adding Griess-Ilosvay reagent to the tube containing nitrate after the reduction of the methylene-blue had been completed, that nitrite had been formed.

On the other hand, there is a possibility that the enzyme activates nitrate so that the active nitrate will reoxidise methylene white, thus retarding the methylene blue reduction [Quastel, Stephenson and Whetham, 1925]. The following experiment was therefore tried. One side of a branched vacuum tube of the type used by Dixon and Tunnicliffe [1927] was filled with 1 cc. enzyme, 0·5 cc. aldehyde and 1 cc. methylene blue. In the other 1 cc. 5 % sodium nitrate was placed. The tube was thoroughly evacuated and the reduction of the methylene blue was allowed to go to completion. The nitrate was then tipped over. If the enzyme activates nitrate, an immediate blueing of the solution ought to occur owing to the reoxidation of methylene white. This did not, however, happen and the retardation of the methylene blue reduction by nitrate is due therefore to its competing as a hydrogen acceptor.

Properties of the enzyme.

The p_H-activity curves for the nitrate and methylene blue reductions are not the same. The nitrate reduction has a wide range from about 3 to 8·6 with an optimum at 5·5. The range with methylene blue is more restricted. In it two factors come into play; namely, the reduction of the dye by the enzyme-aldehyde system in neutral and slightly acid solution and the reduction by the aldehyde in the presence of protein at p_H 8 and over. In the alkaline range acetaldehyde in the presence of protein will reduce methylene blue spontaneously. This is shown by using boiled enzyme and aldehyde at varying hydrogen ion concentrations. No reduction of methylene blue occurs when boiled enzyme and acetaldehyde are incubated below p_H 8. Above p_H 8, however, a reduction occurs. And finally at still higher p_H acetaldehyde alone will reduce methylene blue.

As the following curves show, the methylene blue reduction range for the enzyme aldehyde system is from p_H 5·6 to 7·8. As the protein plus aldehyde does not begin to reduce until p_H 8, there is a discontinuity in the curve. At p_H 7·8, the enzyme becomes least active with methylene blue. When enzyme purified with kaolin is used there is no reduction of methylene blue at all at this p_H.

The hydrogen ion concentrations were determined by the quinhydrone electrode. All experiments given in previous sections of this paper were carried out between p_H 7 and 7·5 where there is a good nitrate reduction and the methylene blue reduction is near an optimum.

The inability of Michlin to obtain the reduction of methylene blue seems to be explained by these p_H-activity curves. It will be seen that at p_H 7·8 the

reaction velocity has a minimum value. In other words, at this hydrogen ion concentration the reduction of methylene blue occurs, at best, slowly, or if the activity of the enzyme is less, not at all. Now Michlin carried out his experiments at p_H 7·8, and it may well be that his inability to obtain any reduction is merely due to this fact. Bach, on the other hand, uses a watery extract of potato, but does not state the p_H of his solutions. Extracts made according to his directions have, however, p_H 5 to 5·3. As seen in the following curves this is too acid for the methylene blue reduction to take place appreciably and explains why Bach failed to get it.

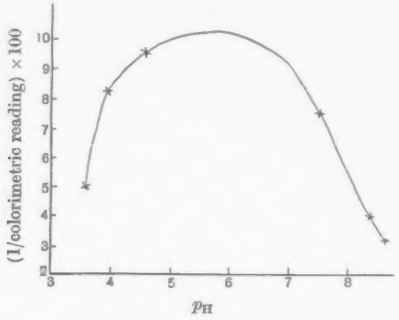

Fig. 2. p_H-activity curve of nitrate reduction.

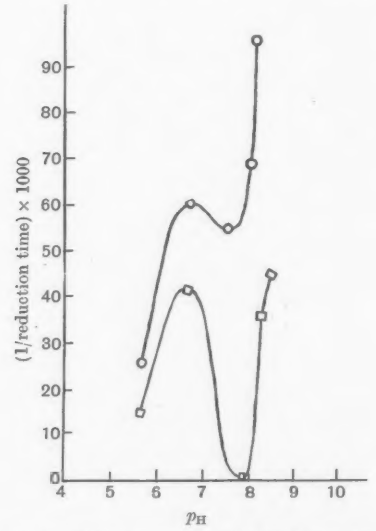

Fig. 3. p_H-activity curve of methylene blue reduction.

□——□ Enzyme treated with charcoal+kaolin.

○——○ Enzyme treated with charcoal.

A preparation of the enzyme was made exactly according to Michlin's directions, and it was found that at p_H 7·3 a good reduction of methylene blue occurred which fell off towards p_H 7·8 showing that this explanation is probably correct.

It should be mentioned here that great care was taken when testing the reduction of methylene blue by any of these preparations to ensure the absence of any substances that might reduce methylene blue spontaneously, or by condensing with the added acetaldehyde, like certain amino-acids. This was done by careful washing of the preparations before the reduction was tested. At p_H 7·5 acetaldehyde and protein will not reduce methylene blue at all in 3 days.

The effect of cyanide was then tried. When M/600 KCN is added the reductions of methylene blue and nitrate are slightly inhibited. The fact that methylene blue reduction is inhibited, and that M/300 KCN produces a marked inhibition of both reductions seems to indicate that KCN is toxic to the enzyme structure itself, and that the reduction of nitrate is not dependent on the presence of traces of iron. If it were, M/600 KCN would probably be more than sufficient to inhibit the reaction completely, as this concentration is effective with other systems dependent on traces of iron. In this respect too the plant is analogous to the animal aldehydase, which, as Bernheim and Dixon [1928] have shown, is unaffected by the addition of cyanide. Both reduction of nitrate and of methylene blue are inhibited to about the same extent, suggesting that the reductions are carried out by the same system.

Exp. 5. 3 cc. buffer p_H 7·3, 1 cc. enzyme, 0·5 cc. 1 % acetaldehyde were placed in each of six tubes with 1 cc. methylene blue or 1 cc. 5 % sodium nitrate. M/300 and M/600 KCN were then added and the reduction time of the methylene blue measured. The nitrate reduction was allowed to proceed 2 hours, and the nitrite formed determined as in the above experiments. Reductions were carried out anaerobically at 37°.

KCN added	Methylene blue reduction time	Nitrate reduction (1/colorimetric reading) × 100
None	80 mins.	50
M/300	200	Slight
M/600	90	45

Precipitants other than ammonium sulphate were tried with potato juice. The juice was precipitated by salts of copper, mercury, lead, iodine, molybdenum, and by phosphotungstic acid. The enzyme is destroyed by all except lead salts. The lead precipitate can be filtered off and obtained in a dry powder which will keep indefinitely. Like the original juice it reduces nitrate alone to a certain extent and very powerfully in the presence of aldehydes. The fact that the lead precipitate to some extent and the ammonium sulphate precipitate to a much less extent reduce nitrate alone is probably attributable to aldehydic substances in the juice which are carried down with the precipitates. One treatment with charcoal in the case of the ammonium sulphate precipitate completely abolishes this reduction. The lead salt being insoluble, it was impossible to treat it with

charcoal. It is used as a suspension in the reduction experiments. It reduces methylene blue alone slowly, and more rapidly in the presence of aldehydes. Treatment with H_2S to break up the lead salt destroys the enzyme.

To prepare the powder, neutral lead acetate is added to the expressed juice until no further precipitation occurs. It is then filtered with suction and washed thoroughly with water. The precipitate is then transferred to an evaporating dish and dried in a vacuum desiccator.

Exp. 6. 1 cc. lead salt suspension and 2 cc. buffer p_H 7·3 were placed in each of four tubes. Tube *A* contained 1 cc. 5 % sodium nitrate, tube *B* the same plus 0·5 cc. 1 % acetaldehyde. Tube *C* contained 1 cc. methylene blue instead of nitrate, and tube *D* the same plus 0·5 cc. acetaldehyde. The reductions were carried out anaerobically at 20°.

Tube	Reduction
A	+ +
B	+ + +
C	270 mins.
D	240

In this preparation of the enzyme also there is a definite correlation between the reduction of nitrate and methylene blue.

Summary.

(1) A method of preparation of the potato aldehyde oxidase is described and the properties of the enzyme are studied.

(2) The aldehyde oxidase will oxidise all aldehydes, but none of the other hydrogen donators tried.

(3) Beside nitrate, methylene blue, Clark's dyes, and quinone are reduced by the enzyme-aldehyde system. The failure of Bach and Michlin to obtain the reduction of methylene blue is explained.

(4) The p_H-activity curves for the nitrate and methylene blue reductions have been obtained.

(5) The action of the enzyme is not dependent on traces of iron.

My thanks are due to Dr Malcolm Dixon for his many suggestions, advice, and help. They are also due to Sir Frederick Hopkins for his interest in the work.

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XLVII. GUANIDINE DETERMINATIONS ON SOME INVERTEBRATES BY A COLORIMETRIC PHOSPHOTUNGSTIC ACID METHOD.

By MAX MAPES ELLIS.

From the Physiological Institute, University of Glasgow.

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Introduction.

It has been suggested [Jaffé, 1906; Paton, 1925] that guanidines may be detoxified in the vertebrate body by an acetylation through which they are transformed into creatine. In view of this possibility and the fact that the muscles of certain invertebrates have been reported as creatine-free [Suzuki, Joshimura and Irie, 1909; Okuda, 1912; Wilson, 1914], guanidine and creatine determinations have been made on molluscan muscle from the limpet, and guanidine determinations on various other invertebrate material.

Method.

1. *Extraction of tissue.* Except as noted under the individual analyses only freshly collected animals were used. The desired tissue was dissected out and minced fine immediately, weighed and put up in absolute alcohol in a ratio of 20 g. of mince to 80 cc. of alcohol. After 48 hours the fluid was drained off and the residue extracted three times with boiling absolute alcohol for three 30-minute periods. After the fluid from the final extraction had been removed, the residue was pressed into a Büchner funnel, sucked dry and then washed with a small quantity of absolute alcohol.

2. *Removal of the undesired extractives.* To the combined alcoholic extracts 15 cc. of distilled water were added and the whole reduced on a water-bath to approximately 10 cc. Sufficient magnesium hydroxide was then added to make the solution barely alkaline to litmus and the evaporation on the water-bath continued to dryness. By this treatment trimethylamine and ammonia are driven off, and methylguanidine is not destroyed [Kuen, 1927].

The residue was then taken up in approximately 30 cc. of absolute alcohol and the insoluble portion centrifuged down. After draining off the supernatant alcohol the precipitate was stirred up with 15 cc. of absolute alcohol and again centrifuged, and the supernatant alcohol added to that from the first centrifuging. The insoluble residue was discarded. The fluid could not be filtered at this stage, as guanidine compounds are adsorbed by filter-paper from both neutral and alkaline solutions. By means of this alcoholic extraction of the dry

residue, arginine (found in crayfish, crabs and lobsters, Ackermann and Kutscher [1907]), lysine (found in crabs, Ackermann and Kutscher; in lobsters, Suzuki, Joshimura and Fuji [1909]), most of the histidine (found in adductor muscle of *Mytilis edulis*, Jansen [1913]), most of the inorganic salts and some of the proteins are left behind in the residue (Guggenheim [1924]; Fränkel [1907]; Thierfelder [1909]).

To this alcoholic extract an equal volume of distilled water was added and the fluid reduced to approximately 10 cc. on the water-bath. The hot liquid was made up to approximately 20 cc. with warm distilled water, and 5 cc. of $2N/3$ sulphuric acid were added. The fluid was then chilled to about 10° and allowed to warm up slowly to room temperature, when sufficient distilled water was added to make an even reading, and the exact volume noted. The liquid was next filtered through a damp, but well-drained filter-paper. This chilling of the acid solution separates the fatty substances and most of the lipochromes, both of which are caught by the wet filter-paper.

An aliquot part (as large as possible) of the clear filtrate was then carefully measured and 4 cc. of 10 % sodium tungstate solution were added for each 20 to 25 cc. of filtrate used. This gives the Folin-Wu [1919] precipitation reagent. If the protein precipitate did not settle quickly 1 cc. of $2N/3$ sulphuric acid and 1 cc. of 10 % sodium tungstate were added. This was repeated until the protein precipitate separated quickly after shaking the fluid, after which 2 cc. of $2N/3$ sulphuric acid in addition to that required for the protein precipitation were added to ensure an acid reaction, and the exact total volume was noted. This Folin-Wu precipitation completes the removal of the protein fraction and the lipochromes.

An aliquot part (as large as possible) of the Folin-Wu filtrate was measured into a flask and approximately 0·5 g. of animal charcoal (purified by acid) added for each 20 cc. of filtrate used. The flask was heated quickly in a water-bath to about 90°, care being taken that the fluid did not boil, and the contents were filtered while hot through a small filter. The flask was rinsed with a small quantity of $2N/3$ sulphuric acid, which was then poured on the filter. To the filtrate a second lot of animal charcoal was added (0·5 g. to 20 cc.) and the process repeated. If the creatine was to be determined the filter-papers and charcoal were preserved in 95 % alcohol, otherwise they were discarded. As each charcoal filtration removes 90 % or more of the creatine, as well as uric acid and phenols [Folin and Dennis, 1916], the double charcoal filtration reduced the creatine content of the filtrate to less than 1 % of the creatine content of the original extract.

After the charcoal filtrations the filtrate was first neutralised with 10 % sodium hydroxide, then made barely acid to litmus with as little hydrochloric acid as required, after which the fluid was evaporated to dryness on a water-bath. These adjustments of the reaction of the filtrate are necessary because either free sulphuric acid or alkali may decompose some of the guanidine on concentration. As the small quantity of excess hydrochloric acid required to

bring the solution to an acid reaction after neutralisation with sodium hydroxide is volatile, the solution may be evaporated on a water-bath with safety.

The residue from this evaporation was taken up in not more than 5 cc. of 5 % sulphuric acid, transferred to a centrifuge tube and the insoluble material allowed to settle. It is essential that the volume be kept as small as possible at this stage on account of the phosphotungstic acid precipitation. After standing for a few minutes, but without removing any of the material from the tube, 30 % aqueous phosphotungstic acid solution was added drop by drop until two drops gave no additional clouding after shaking the fluid at the top of the tube. The tube was then placed in an ice-chest, or otherwise kept cold (below 10°) for 18 hours, after which the preparation was centrifuged. The precipitate consisted essentially of guanidine, methylguanidine and betaine phosphotungstates, together with the small insoluble residue which formed on drying down the Folin-Wu filtrate.

3. *Determination of guanidine.* The final determination of the guanidines was made by means of the nitroprusside reaction, and Marston's reagent [1924] as modified by Weber [1927] was used. This reagent is prepared about 30 minutes before it is required by mixing equal volumes of 10 % solutions of sodium nitroprusside, potassium ferricyanide and sodium hydroxide. The stock solutions of nitroprusside and ferricyanide should be kept in the dark and made up fresh every few days. The nitroprusside reagent was chosen because of the presence of betaine phosphotungstate in the precipitate with the guanidines, and because of the small quantities of guanidines to be determined. As the solubility of betaine phosphotungstate in methyl alcohol is twice that of creatine phosphotungstate [Drummond, 1918], a gravimetric separation of guanidine and betaine phosphotungstate on the basis of their solubilities in methyl alcohol, like that used by Kühnau [1926] in his separation of guanidine and creatine phosphotungstates from vertebrate blood, is undesirable. It was found that betaine did not give a colour reaction with the nitroprusside reagent and that the small quantities of betaine carried over in the methyl alcohol extraction of the phosphotungstic precipitate did not influence the colour reactions of the guanidines.

After draining off all the fluid from the phosphotungstic precipitate 2 cc. of methyl alcohol (acetone-free) were added, the precipitate was mixed with the alcohol, and the insoluble portion centrifuged down. The methyl alcohol solution was drained off into an evaporating dish, the tube washed with 1 or 2 cc. of methyl alcohol (care being taken not to disturb the precipitate) and the washing added to the first methyl alcohol solution. This methyl alcohol contained the guanidine and methylguanidine phosphotungstates and a small quantity of betaine phosphotungstate. The recovery of the guanidines was quite complete, for 29·2 parts of guanidine phosphotungstate are soluble in 100 parts of methyl alcohol [Drummond, 1918] and 16 parts of methylguanidine phosphotungstate [Kühnau, 1926], as against 1·2 parts of betaine phosphotungstate and 0·6 part of creatine phosphotungstate [Drummond, 1918].

To the alcoholic solution of the phosphotungstate four volumes of distilled water were added (this produces a clouding due to the formation of a partial precipitate of the phosphotungstates), and the whole was reduced to about one-half the original volume on a water-bath. This fluid was then first neutralised with 10 % sodium hydroxide and next made just acid to litmus with as little hydrochloric acid as required, and the evaporation continued on the water-bath to dryness. This step is essential, as methyl alcohol gives a colour reaction with the nitroprusside reagent.

The residue was taken up in exactly 5 cc. of 2 % sodium hydroxide and without removing the solution from the evaporating dish exactly 1 cc. of the nitroprusside reagent was added. At the same time the nitroprusside reagent was added to the standard (see below) and the readings were made with a colorimeter after 10 to 15 minutes. As this method does not differentiate between guanidine and methylguanidine, and as the colorimetric values of the phosphotungstates of these two radicles are not the same, the final results have been reckoned both as guanidine and methylguanidine, the true values lying somewhere between these limits (see Table III).

4. *Standard for guanidine determinations.* As it was found that the shade of colour produced by guanidine and methylguanidine phosphotungstates with the nitroprusside reagent differed from the shade produced by the hydrochlorides of these radicles with that reagent, standards were first prepared from weighed quantities of repurified guanidine phosphotungstate. As this compound is difficult to prepare and does not keep well, after the preliminary standardisations were completed, the standards were prepared directly from dry, crystalline, guanidine hydrochloride (Merck) which has one molecule of water of crystallisation, as follows: to 1 cc. of 1 % aqueous solution of guanidine hydrochloride 1 cc. of 7 % phosphotungstic acid (Merck) was added and the resulting turbid fluid well shaken; after 5 minutes 3 cc. of 3·5 % sodium hydroxide solution were added and the mixture was again shaken until the precipitate was dissolved; to this solution 1 cc. of nitroprusside reagent was then added. The full colour develops in about 4 minutes and the standard is good for 30 minutes or longer, but readings were generally made after 10 to 15 minutes. This standard solution contains 5·202 mg. guanidine, and, as it contains the same amount of sodium hydroxide as the solution of the unknown, direct comparisons were made in the colorimeter. As guanidine hydrochloride is hygroscopic, samples used for standards were dried in a desiccator before being weighed. The aqueous solution of guanidine was not kept for more than 24 hours (*vide infra*).

Methylguanidine phosphotungstate when precipitated from methylguanidine hydrochloride (British Drug Houses, highest purity) gave approximately 3·2 times as much colour as guanidine phosphotungstate with the nitroprusside reagent. The quality of the colour, however, was the same as that given by guanidine phosphotungstate so that readings could be made against either a methylguanidine standard or a guanidine standard. Dimethylguanidine phos-

photungstate, when precipitated from dimethylguanidine hydrochloride (British Drug Houses, highest purity), gave a distinctly different shade of red with the nitroprusside reagent from that produced by either guanidine or methylguanidine. Accordingly this substance had to be compared with special standards.

5. *Limits of guanidine precipitation by phosphotungstic acid.* As this method depends critically on the completeness of the precipitation of the guanidines by phosphotungstic acid, and as two of the recent methods for guanidine determinations have also used phosphotungstic acid precipitations [Kühnau, 1926; Kuen, 1927], a series of recoveries of guanidines by phosphotungstic acid precipitation from known solutions of guanidines was studied.

The solutions of guanidine, methylguanidine and dimethylguanidine hydrochlorides were made up in 5 % sulphuric acid, as optimum precipitation with phosphotungstic acid is obtained from such a solution [Gulewitsch, 1899; Demjanowski, 1912; Drummond, 1918], and 30 % phosphotungstic acid was added in slight excess of the amount required for complete precipitation on the basis of molecular weights. These precipitation tests were made at two different temperatures, "room temperature"—around 18° dropping to possibly 10° at night, and "ice-chest temperature"—a constant temperature of 7°. The

Table I. *Averaged data for guanidine precipitations by phosphotungstic acid from* 5 % *sulphuric acid solutions.*

Compound in solution	Amount of salt in 1 cc. mg.	Amount of organic base in 1 cc. mg.	Base recovered by precipitation: Room temp. 18°–10° %	Base recovered by precipitation: Ice-chest 7° %
Guanidine hydrochloride (Merck)	4	2·08	100	100
	2	1·04	100	100
	1	0·52	91	97
	0·6	0·31	85	95
	0·5	0·26	Trace	60
	0·4	0·21	None	22
	0·2	0·10	None	None
Methylguanidine hydrochloride (B.D.H., highest purity)	2	1·33	100	100
	1	0·67	100	100
	0·6	0·40	100	100
	0·5	0·33	100	100
	0·4	0·27	79	100
	0·2	0·13	None	95
Methylguanidine hydrochloride (B.D.H., highest purity) kept as a 0·5 % aqueous solution for 1 week before testing	2*	1·33*	—	23
	1*	0·67*	—	21
	0·6*	0·40*	—	11
	0·5*	0·33*	—	None
	0·4*	0·27*	—	None
Dimethylguanidine hydrochloride (B.D.H., highest purity)	2	1·40	100	—
	1	0·70	93	—
	0·6	0·42	82	—
	0·5	0·35	71	—
	0·4	0·28	66	—
	0·2	0·14	None	—

Probable error of the determinations does not exceed ±3 %.

* Amounts which should have been present had there been no decomposition of the salt in the aqueous solution.

precipitations were made from 5 cc. samples of various concentrations and in each case the tubes were allowed to stand for 18 hours before centrifuging. The determinations of the recoveries were made gravimetrically, colorimetrically and by titration (a method to be described elsewhere), and the averaged data are summarised in Table I.

From this table it is evident that the precipitation is more complete at a constant low temperature than at room temperature and that the differences are sufficient to make comparisons of determinations at room temperature unreliable for quantitative work. At ice-chest temperature the limits of "complete" precipitation, considering the probable error, seem to be near 0·75 mg. of guanidine and 0·20 mg. of methylguanidine per cc., and no determinations of guanidines from tissues have been considered as accurate in this report when less than 0·50 mg. of guanidine or 0·25 mg. of methylguanidine per cc. were found in the concentrate as taken up finally in 5 % sulphuric acid. One series of guanidine precipitations, not listed in the data, was made at − 6°, and, as the recoveries did not differ essentially from those at + 7°, ice-chest temperature or 7° was adopted for this work.

In considering the reliability of standards one lot of 0·5 % aqueous methylguanidine solution was kept for a week at room temperature, sufficient 12·5 % sulphuric acid was then added a drop at a time to bring the whole up to 5 % sulphuric acid, after which the usual phosphotungstic acid precipitations were carried out at ice-chest temperature. This check series has been included in Table I, as the recoveries show that a 0·5 % aqueous solution of methylguanidine is unstable. Accordingly guanidine solutions more than 24 hours old were not used as standards.

An example of the result obtained in the determination of guanidine added to ox heart is given in Table II.

Table II. *Example of guanidine recovery by method described.*

Material	Guanidine hydrochloride added mg.	Guanidine added mg.	Guanidine found mg.	Guanidine recovered %
50 g. ox heart	None	None	0·648	—
50 g. ox heart	4·675	2·432	2·951	94·64

GUANIDINE CONTENT OF TISSUES AND ANIMALS EXAMINED.

In Table III the data from the various sets of determinations on invertebrate tissues have been summarised together with three determinations on vertebrate muscle. Owing to the small size of the invertebrates used it was not possible to get samples of muscle tissue alone large enough for guanidine determinations (samples of 100 g. or more were used), except in the case of the limpet. As the large muscular foot of this animal can be dissected away quickly and consists of almost pure muscle tissue, large samples were easily obtained. Two sets of limpet samples were extracted, the muscle tissue for one set ((*a*) in Table III) being taken from freshly collected animals within a few

minutes after they were removed from the rocks on which they were living, while the muscle tissue for the other set ((*b*) in Table III) was dissected from limpets which had been removed from the rocks and kept alive and unattached in the laboratory for 24 hours before the dissection was begun. When the limpet is unattached the foot-muscle contracts violently and is drawn up into a central, saucer-shaped mass in a state resembling tetanic contraction. As the guanidine content of blood from man and various other mammals is reported as high in certain tetanies [Paton, 1925; Kühnau, 1926] it seemed desirable to compare these molluscan muscles following this natural "tetany" with normal muscles from freshly collected limpets, but neither creatine nor guanidines were detected in either series of limpet muscles. These results agree with the previous findings of Wilson [1914] in the scallop and periwinkle for creatine. Betaine was present in considerable quantity in the limpet muscle and was recovered as a phosphotungstate, although no quantitative determinations for betaine were made. Considering these results on muscle from the limpet in connection with the suggestions of Jaffé [1906] and Paton [1925] that guanidines may be detoxified into creatine, two interpretations are possible, either that there are no guanidines produced by the molluscan muscle to be acetylated to creatine, or that the guanidines if formed in molluscan muscle are rapidly and completely transformed into some other compound. Betaine may have some significance in this connection, as Hunter [1922] has suggested betaine as possibly the evolutionary precursor of creatine.

Table III. *Guanidine content of tissues and animal examined.*

Systematic name	Common name	Part used	Guanidine per 100 g. of fresh tissue: As guanidine mg.	Guanidine per 100 g. of fresh tissue: As methyl-guanidine mg.
Coelenterata—				
Metridium dianthus	Sea anemone	Entire animal	None	None
Echinodermata—				
Asterias rubens	Common starfish	Entire animal	None	None
Mollusca				
Patella vulgata (*a*)	Limpet	Foot-muscle	None	None
,, ,, (*b*)	,,	,,	None	None
Purpura lapillus	Dog whelk	Foot-muscle plus some viscera	5·01	1·67
Arthropoda				
Calanus helgolandicus	Copepod	Entire animal	10·27	3·21
Balanus balanoides	Acorn barnacle	,, ,,	None	None
Ligia oceanica	Marine isopod	,, ,,	12·87	4·01
Gammarus marinus	Amphipod	,, ,,	None	None
Carcinus moenas	Shore crab	Legs and claws	2·62	0·82
Vertebrata	Ox	Skeletal muscle	2·20	0·69
	,,	Heart muscle	1·29	0·43
	Dog (*pt.*)	Skeletal muscle	2·12	0·66

(*a*) Muscle taken from freshly collected animals. (*b*) Muscle taken from animals in which the foot had been strongly contracted for 24 hours. (*pt.*) Parathyroidectomised dog.

All invertebrates were collected October 30 and 31, and November 1, 1927, except *Calanus*, which was taken May 25, 1925. Samples averaged 100 g. or more.

In preparing the tissue from the dog whelk for extraction the muscular foot was dissected out as in the limpet, but owing to the smaller size of the whelk and the differences in the arrangement of the viscera the samples of whelk tissue were not pure molluscan muscle, but muscle plus some viscera. The exact amount of the latter was difficult to estimate, but certainly did not exceed 20 % of the material. The analyses of these samples showed the presence of guanidines to the extent of 1·67 mg. of methylguanidine per 100 g. of tissue. Of course it must be recognised that these figures are open to two objections. The determinations may not represent guanidines actually produced in the body of the whelk, as the guanidines found may have been present in either plant or animal tissue included in the intestinal detritus with the viscera which were not completely dissected away, although certainly the total amount of such material was not large. It is also possible that the substances determined were not guanidines but some other unknown substances having the same separation and precipitation characteristics as the guanidines and giving the nitroprusside reaction.

Similarly the sample of tissue from the shore crab, although very largely muscle, contained some other tissues, as they were prepared by grinding the legs and claws into a fine mince for extraction. By weighing, drying and ashing various samples, the actual weight of the tissue extracted independent of the shell was computed and the values for the guanidines found have been reckoned on the basis of the weight of the fresh tissue alone. Suzuki, Joshimura and Irie [1909] in their extractions of the fresh shell of lobsters report 0·042 % of lysine, the other extractives being present in traces only, and, as lysine was excluded from the extractions of crab material discussed above, it seems unlikely that the presence of the shell materially affected the extraction of the other tissue.

For the other extractions entire animals were minced, but the findings give the guanidine content of the whole animal on the basis of the weight of the fresh tissue alone, corrections being made for the weight of the shell or exoskeleton. Two of the species used, the shore isopod and the pelagic copepod, contained guanidines or guanidine-like substances, the other species examined did not. Here again the possibility that these findings from whole animals may have been based on guanidines present in the intestinal contents rather than on guanidines produced by the animals themselves must be borne in mind.

The three determinations on vertebrate muscle included in Table III are taken from another series of analyses bearing on other experiments, and are added to show that the method as described can be applied to vertebrate muscle as well. The ox tissues examined were ordinary butcher's beef and ox heart, and the dog muscle was obtained from a parathyroidectomised dog which had been given calcium so that its tetany was reduced. All these values for guanidine are much lower than those reported by Smorodinzew [1914] for sheep flesh, but as he used a baryta-silver method it is possible that some of the creatine of the muscle was changed into methyl-

guanidine by the reagents [Ewins, 1916], and included in the determinations as guanidine. The results for vertebrate muscle given in Table III, however, are higher than those given by Weber [1927] for normal human blood (0·07–0·15 mg. per 100 cc.) or even for hypertension cases (0·3–0·6 mg. per 100 cc.).

SUMMARY.

1. A colorimetric phosphotungstic acid method for the determination of guanidines in invertebrate and other tissues is described.

2. Pure samples of molluscan muscle from freshly collected limpets and from unattached limpets, in which the foot muscle had been in prolonged contraction, were found to be both creatine- and guanidine-free.

3. Guanidines or substances giving the separation, precipitation, and nitroprusside reactions of guanidines, were found in four invertebrates (the dog whelk, *Purpura lapillus*; the pelagic copepod, *Calanus helgolandicus*; the marine isopod, *Ligia oceanica*; and the shore crab, *Carcinus moenas*), in which animals tissues other than muscle alone were extracted.

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XLVIII. CHEMICAL CHANGES IN MUSCLE.

PART II. INVERTEBRATE MUSCLE.
PART III. VERTEBRATE CARDIAC MUSCLE.

By ERIC BOYLAND.

From the Department of Physiology, the University, Manchester, and the Laboratory of the Marine Biological Association, Plymouth.

Report to the Medical Research Council.

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PART II. INVERTEBRATE MUSCLE.

FLETCHER AND HOPKINS [1907] in their researches on lactic acid in amphibian muscle found that a fairly definite amount of lactic acid was produced on stimulation to fatigue and that a larger amount accumulated when the muscle was allowed to pass into rigor. Later, Meyerhof [1920, 1921] demonstrated that under anaerobic conditions this lactic acid production was accompanied by an equivalent decrease in the glycogen present. If the muscle was suspended in an alkaline phosphate solution all the glycogen became converted into lactic acid.

It has been possible to demonstrate that this change occurs in most muscles of invertebrate animals; though the source of the lactic acid may not always be glycogen alone and the quantitative aspect of the change varies in different muscles. From this it appears probable that the theories of muscular activity based on experiments with muscles of vertebrate animals can be applied almost universally.

In *Octopus* muscle Henze [1904] found no glycogen and only a very small amount of lactic acid, which, from the determination of the water of crystallisation of the zinc lactate obtained, he considered to be inactive. As the water content of zinc lactate often shows great variation, the evidence put forward on this basis is not conclusive. Later, Henze [1909] found moderately large amounts of glycoprotein in molluscan muscle; he suggested that this formed the reserve material of these animals and that its breakdown was responsible for muscular activity. Still later, however, Starkenstein and Henze [1912] were able to find glycogen in the liver and muscles of *Octopus* and *Aplysia*. The greater quantity was contained in the muscles. Lobster muscle appears to convert glycogen into lactic acid, as Ritchie [1927] isolated zinc sarcolactate from lobster muscle which had been suspended in a solution of disodium

hydrogen phosphate. At the same time, qualitative examination showed that glycogen had disappeared. Weinland [1901] after investigating the parasitic worm, *Ascaris*, considered that it lived anaerobically and transformed glycogen into valeric acid and carbon dioxide. Slater [1925], however, found that *Ascaris* could not live anaerobically for very long and that the valeric acid was produced by bacteria accompanying the worm. Thus it seemed probable that invertebrate animals used the same metabolic processes as vertebrate animals, in those cases which had been examined.

Nevertheless, several workers had analysed the adductor muscle of lamellibranchs, but none claimed to have found lactic acid. On the other hand, large quantities of glycogen have often been obtained from this muscle. Thus Chittenden [1895] found 2·0 % of glycogen in the muscle of *Pecten irradiens*, Bradley and Kellersberger [1912] found 4·6 % in *Pecten* muscle and Albrecht [1920] obtained 1·9 % from the *Pismo* clam. Jansen [1913], Ackerman [1921], and Daniel and Doran [1926], isolated mytilitol from *Mytilus* muscle, but do not mention lactic acid, though it is probable that attempts had been made to detect it. Kestner and Plaut [1911], quoting Weinland, say that the freshwater mussel, *Anodon*, expends only about two Calories of energy in 8 months. With this low expenditure of energy, it is improbable that there is ever much accumulation of lactic acid in the sedentary molluscs. This low metabolism increases the difficulty of investigation of the chemical changes occurring in the muscles of invertebrate animals.

Crustacea.

Experimental.

The abdominal muscle of the decapod crustacean, *Palinurus vulgaris*, or the crawfish, was first examined. Healthy animals were taken from the aquarium tanks. After destroying the supra- and sub-oesophageal ganglia, the abdomen was removed from the remainder of the body and the muscle taken out, which could not be done without considerable stimulation, with the result that it was impossible to obtain samples of the muscle in a resting condition. The abdomen of the crawfish is a powerful weapon of attack and contains large masses of clean, white, striated muscle; this was carefully separated from chitinous and other matter. A sample was immediately treated with cold alcohol, as described by Fletcher and Hopkins [1907], for the estimation of the resting value of lactic acid, another sample stimulated to fatigue (*i.e.* till stimulation produced no visible response) was extracted with alcohol in the same way, while other samples were allowed to stand in the presence of chloroform for 6 hours to produce rigor, and in a solution of disodium hydrogen phosphate for 24 hours to allow of the maximum formation of lactic acid. The muscle was then analysed for glycogen, lactic acid and lower carbohydrates by methods described by the writer [1928]. Some results are given in Table I. As a check on the estimation of lactic acid, determinations were made by the

oxidative method, using steam distillation as generally employed and by the gravimetric method of isolating the zinc salt after ethereal extraction of the acid. The excellent agreement between the two methods indicates that the oxidative method gives reliable results on application to crustacean muscle. The figures for glycogen are always calculated on the formula of the hydrate $(C_6H_{10}O_5)_n \cdot nH_2O$ which can easily be isolated [see Slater, 1924] and, being a carbohydrate of the same empirical formula as glucose or lactic acid, affords a very convenient form in which to state the results.

Table I. Palinurus vulgaris *abdominal muscle.*

State	Weight of muscle g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Total %
		Normal animals.			
Rest	20·0	0·14	0·09	0·07	0·30
Fatigued	20·0	0·16	0·05	0·08	0·29
Rigor	20·0	0·18	0·02	0·09	0·29
Phos. max.	20·0	0·21	0·02	0·07	0·30
Phos. max.	100·0	0·205 (by Fletcher and Hopkins's method)			
Rest	20·0	0·18	0·15	0·08	0·41
Fatigued	20·0	0·25	0·10	0·07	0·42
Rigor	20·0	0·34	0·02	0·08	0·44
Phos. max.	20·0	0·38	0·03	0·09	0·50
		Animal with soft shell some days after moult.			
Rigor	4·20	0·12	0·06	0·03	0·21

The results demonstate the interdependence of lactic acid formation and the glycogen utilisation. There is a quantitative conversion of glycogen into lactic acid, as Meyerhof [1920] had previously found in frog muscle. The increase of lactic acid, in fatigue and in rigor, though smaller in amount than is usually the case in vertebrate animals, indicates that lactic acid production is closely related to activity in this muscle. Further experiments were carried out on the abdominal muscle of the common lobster (*Homarus vulgaris*) which was treated in a similar way to that already described. It might be noted that the two moulting lobsters examined were both killed while leaving their old shells, that is, during the actual moult and not during the subsequent hardening of the new shell. The results shown in Table II indicate the conversion of glycogen into lactic acid; they are very similar to those obtained for the muscle of *Palinurus*. Experiments on the small active prawn, *Leander serratus*, also gave similar results, shown in Table III. The glycogen values of the prawn are probably below their usual values as the animals were sent from Plymouth to Manchester without being supplied with food. This would cause the glycogen reserves to be used up, and almost all the glycogen is converted into lactic acid on stimulation to fatigue. Very little more lactic acid is formed either in rigor or on suspension in a phosphate buffer solution.

Glycogen, lactic acid and lower carbohydrates were examined in the heart muscle of *Homarus* and *Palinurus*. The shell of the animal was cut away above the heart, which was then dissected out and treated in the usual way. Owing to the small amount of tissue available, the estimations of glycogen and lower

Table II. Homarus vulgaris *abdominal muscle.*

State	Weight of muscle g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Total %
		Normal animals.			
Rest	12·12	0·03	0·05	0·08	0·16
Fatigued	22·32	0·11	0·03	0·03	0·17
Phos. max.	12·75	0·13	0·02	0·03	0·18
Rest	10·68	0·04	0·05	0·05	0·14
Fatigued	12·42	0·10	0·02	0·05	0·17
Phos. max.	16·92	0·11	0·02	0·06	0·19
Rest	23·50	0·05	0·11	0·05	0·21
Fatigued	14·02	0·08	0·05	0·07	0·20
Phos. max.	32·33	0·11	0·01	0·08	0·20
Rest	16·03	0·05	0·20	0·05	0·30
Fatigued	14·34	0·10	0·16	0·06	0·32
Phos. max.	29·64	0·22	0·02	0·06	0·30
Rest	24·65	0·09	0·10	0·04	0·23
Fatigued	12·87	0·13	0·05	0·06	0·24
Rigor	13·60	0·14	0·03	0·06	0·23
Phos. max.	12·02	0·15	0·02	0·07	0·24
		Female animals with eggs.			
Rest	25·74	0·13	0·18	0·04	0·35
Fatigued	25·04	0·16	0·13	0·03	0·32
Rigor	16·63	0·27	0·03	0·04	0·34
Phos. max.	17·94	0·28	0·02	0·07	0·37
Rest	20·0	0·17	0·17	0·08	0·42
Fatigued	20·0	0·25	0·09	0·07	0·41
Rigor	20·0	0·31	0·02	0·09	0·42
Phos. max.	20·0	0·37	0·005	0·09	0·46
		Moulting animals.			
Rest	10·0	0·06	0·14	0·19	0·39
Phos. max.	40·0	0·25	0·03	0·17	0·45
Rigor	50·0	0·19	0·28	0·12	0·59

Table III. Leander serratus *abdominal muscle.*

State	Weight of muscle g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Total %
Rest	0·76	0·13	0·11	0·09	0·33
Rest	1·54	0·14	0·11	0·05	0·30
Fatigued	1·93	0·15	0·07	0·04	0·26
Fatigued	2·35	0·17	0·06	0·03	0·26
Rigor	2·27	0·16	0·07	0·03	0·26
Rigor	1·71	0·06	0·08	0·04	0·18
Phos. max.	2·73	0·14	0·05	0·03	0·22
Phos. max.	1·26	0·13	0·08	0·03	0·24
		Experiments on one sample.			
Rest	0·84	0·13	0·07	0·07	0·27
Fatigued	0·98	0·23	0·04	0·03	0·30
Rigor	1·52	0·14	0·07	0·04	0·25
Phos. max.	1·18	0·23	0·06	0·03	0·32

carbohydrates are not so reliable as those made on larger samples. Estimations were carried out on the abdominal muscle of the same animal, so as to allow of comparison of heart and skeletal muscle of one animal. The similarity of the two forms of muscle can be seen from Table IV.

Table IV. *Comparison of crustacean heart and skeletal muscle.*

Muscle and state	Weight of muscle g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Total %
		Palinurus vulgaris.			
Abdomen					
Rest	3·08	0·08	0·11	0·01	0·20
Heart					
Rest	0·50	0·16	0·24	0·02	0·42
Fatigued	0·50	0·24	0·14	0·03	0·41
Rigor	0·50	0·15	0·17	0·02	0·34
Phos. max.	0·50	0·26	0·05	0·04	0·35
Abdomen					
Rigor	4·20	0·22	0·06	0·03	0·31
Heart					
Rigor	0·27	0·22	0·11	0·03	0·36
Phos. max.	0·32	0·38	0·04	0·03	0·45
		Homarus vulgaris.			
Abdomen					
Rest	4·17	0·08	0·12	0·03	0·23
Heart					
Rest	0·27	0·05	0·25	0·06	0·36
Rigor	0·27	0·28	0·04	0·08	0·40
Abdomen					
Rest	1·50	0·09	0·17	0·02	0·28
Heart					
Rest	0·58	0·08	0·60	0·05	0·73
Heart					
Rest	0·23	0·17	0·39	0·05	0·61
Fatigued	0·28	0·30	0·25	0·06	0·61
Rest	0·415	0·18	0·39	0·02	0·59
Phos. max.	0·480	0·56	0·04	0·03	0·63

Discussion.

Crustacean muscle converts glycogen into lactic acid in activity, as has been demonstrated in muscles of vertebrate animals by previous workers, but the glycogen content of the muscle and the lactic acid formed in fatigue, in rigor or in buffer solutions are all less than are usually found in amphibian or mammalian muscle. The glycogen content of the muscles of different individuals seems to vary to a greater extent than it does in normal vertebrate animals. It would seem that the muscle of invertebrates acts as a store of glycogen; while higher animals store glycogen and fat in the liver, which keeps the muscle glycogen up to a fairly constant level. In the crustacea, the glycogen content of the muscle varies with reproductive cycles; it can be seen from Table II that breeding and moulting lobsters had definitely higher glycogen contents than normal lobsters. The increase in the case of breeding lobsters is possibly due to storage, in order to provide energy for the period during which the animal is less active.

A marked increase in glycogen and in lower carbohydrates is also found in the two moulting lobsters which were examined. On the other hand, a *Palinurus*

with a moderately soft shell, which had probably left its old shell some time before examination, had almost the normal amount of glycogen, having used up its excess stores in secreting the chitin of the new exoskeleton. This leads one to suppose that the growth cycle of crustacea is divisible into three stages; a slow accumulation of calcium phosphate, fat and glycogen, followed by the moult in which glycogen is rapidly used up to form chitin; finally, the soft chitinous shell is hardened by deposition of calcium phosphate. Paul and Sharpe [1915] in an investigation of calcium deposition in crustacea found that some crustacea store calcium phosphate and fat in the hepato-pancreas, previous to the moult. They estimated that a tenfold increase in blood volume may occur in moulting crustacea. That there is some increase can easily be seen; for if the soft shell is pierced, the blood rapidly escapes as if it were under considerable pressure, though it is doubtful if the increase is as great as tenfold. The increase in the amount of blood expands the new soft shell so that the new shell is larger than the old one. The extra blood also probably helps in the transport of calcium phosphate from the hepato-pancreas to the shell, where it is deposited. On examination of the results of the analyses of glycogen it seems that glycogen is stored before the moult or else it is derived from fat which had been stored. There is also an increase in lower carbohydrates which is not explained by the increase in volume of blood, as the amount of lower carbohydrates is greater than the usual blood-sugar of crustacea. As calcium phosphate is being deposited, it might be possible for the blood to contain hexosephosphates such as those concerned in bone formation of vertebrate animals, but this is not supported by examination of the lower carbohydrates extracted from the moulting animals, which are found to have the same reducing power before and after acid hydrolysis. On the other hand, a similar extract from normal frog muscle has increased reducing power after acid hydrolysis. The fact that the lower carbohydrates had fallen to the normal value in the case of the soft-shelled *Palinurus* examined (Table I) indicates that the increase in lower carbohydrates is related to chitin formation rather than to calcium deposition, as this animal would have deposited its chitin but would still be depositing calcium phosphate and calcium carbonate. The increase in glycogen content on moulting is in agreement with the early work of Kirch [1886] which, however, was not available. Kirch found 0·08 % of glycogen in normal crawfish (*Palinurus*), but 0·40 % just before the moult and 0·82 % during the moulting period. Hoet and Kerridge [1926] say that they were unable to confirm this; they found very little glycogen and acid production in soft-shelled crustacea.

It can be seen from their results that the glycogen content of the soft-shelled animals is little lower than that obtained on several normal lobsters in fatigue and in rigor (cf. Table II). If glycogen is expended in chitin formation, one would expect a low glycogen content in soft-shelled animals which have just laid down the chitin of their new shell. The soft-shelled animals would not be able to get much food, and *Carcinus* and *Cancer*, on which most of the experiments of Hoet and Kerridge were carried out, are very active animals which

would probably use up glycogen in efforts to escape. The results of Hoet and Kerridge were obtained on soft-shelled animals which had passed the stage of chitin deposition, involving changes in carbohydrates, and they show that glycogen is used up in this process.

The soft-shelled animals would be hardening their shells by depositing calcium phosphate and calcium carbonate in the newly formed chitin. Paul and Sharpe indicate that this hardening may take up to 2 months, during the early part of which the animal is inactive. This deposition of calcium salts in the shell would account for the alkalinity of the muscles of the soft-shelled animals examined by Hoet and Kerridge. These determinations show the muscles of soft-shelled animals 20 hours after death to be more alkaline than those of freshly killed animals as determined later by Furusawa and Kerridge [1927]. The increased alkali or buffer in the blood during hardening of the shell would neutralise the acid produced by the muscle in rigor. Thus all the facts support the hypothesis that the moulting cycle of crustacea involves (1) a slow accumulation of calcium phosphate and fat in the hepato-pancreas and glycogen in the muscles, (2) a rapid formation of a soft chitinous shell, with utilisation of glycogen, and (3) a slow hardening of the shell by calcium deposition.

Analyses of crustacean heart muscle indicate that it generally contains more glycogen than the skeletal muscle of the same animal, and that all the lactic acid produced can be accounted for by the glycogen originally present in the muscle. Katz and Long [1925] in comparing mammalian cardiac and skeletal muscle found greater lactic acid production in skeletal than in cardiac muscle and that the heart muscle could produce more lactic acid than the equivalent of the glycogen present. Thus, both in the amount of lactic acid produced in activity and in the source of the acid, crustacean heart muscle resembles skeletal muscle rather than the specialised mammalian cardiac muscle. This is in agreement with histological evidence, for crustacean heart muscle appears to be striated like crustacean skeletal muscle.

Mollusca.

Experimental.

Although cephalopods and gastropods were examined, most experiments were made on the lamellibranch, *Pecten*. The *Pecten* or scallop is a bivalve more active than other molluscs with the exception of the cephalopods. It swims through the water by opening and closing the valves of its shell. Attached to the shells and causing their movement are two adductor muscles. One is large, translucent in appearance and quick in action, while the other is small, opaque and slow acting. The former is called the large adductor muscle, the latter the small adductor or "catch" muscle. The large muscle causes the quick movements of the shell which propel the animal through the water, the small merely serves to keep the valves closed. For experiments the muscles were obtained by first cutting the muscle and the hinge, so as to remove one valve of the shell:

the muscles were separated for analysis. It was almost impossible to remove the muscle without stimulation, sometimes to such an extent with *Pecten maximus* that they were incapable of further activity immediately after removal. The large muscle was investigated first in several species of *Pecten* and was found to contain large and varying amounts of glycogen. The glycogen present probably varies with reproductive changes, as was found in the lobster; an attempt to follow this variation was made in the case of *Pecten opercularis*. In spite of the large amount of glycogen, very little lactic acid was formed under any conditions. Considerable difficulty was encountered in proving the presence of lactic acid, even qualitatively, owing to the relatively large amounts of other substances such as dextrins, mytilitol, taurine and succinic acid. On account of the succinic acid it was not possible to isolate pure zinc lactate, and estimations of lactic acid by the method of Fletcher and Hopkins gave high results owing to considerable contamination of the zinc lactate with zinc succinate. The oxidative method of estimation of lactic acid gave much lower results which probably approached the true value. By taking a large quantity of muscle and carefully removing other substances, the presence of lactic acid was indicated by the Hopkins thiophen reaction and it was estimated by oxidation to acetaldehyde in a current of steam followed by iodometric determination of the acetaldehyde produced. The use of large amounts of muscle, however, led to incomplete extraction of the acid and probably erroneous low results. To illustrate the difference in results obtained by the two methods of estimation, one experiment may be cited. 500 g. of the large adductor muscle of *Pecten opercularis* gave 325 mg. of lactic acid as determined by the method of Fletcher and Hopkins and only 128 mg. as determined by the oxidative method. The zinc lactate obtained after ethereal extraction of the acid contained 37·1 % zinc oxide, instead of the theoretical amount of 33·8 %. Three species, *Pecten opercularis*, *Pecten maximus* and *Pecten varius*, were examined at Plymouth. The larger *Pecten magellanicus* was obtained by Mr A. D. Ritchie at the Atlantic Biological Station, St Andrews, New Brunswick, Canada. The muscle of the last species, after standing in phosphate solution, was extracted in Canada and the lactic acid estimated in the extract at Manchester. The results obtained from experiments on the large adductor muscle are given in Table V, the muscle being treated in a similar manner to that described in the case of crustacean muscle.

In the last experiment with *Pecten opercularis*, in which the muscle contained large quantities of glycogen, it is probable that some of the glycogen became converted into dextrin and so escaped estimation. Bradley and Kellersberger [1912] found that erythrodextrins were formed in *Pecten* muscle containing 4·6 % glycogen. This change to dextrins appeared to occur in many samples which were not in a perfectly fresh condition.

After lactic acid had been identified and estimated in the large adductor muscle, the small adductor muscle was investigated, in *P. opercularis* and *P. maximus*. Only small amounts of this muscle were available, as individuals

of *P. maximus* which may contain 20 g. of the large adductor muscle often yielded less than 1 g. of the small muscle. The muscles obtained from several animals were mixed and examined in the same way as the large muscles. The lactic acid determinations were all made by the oxidative method; the results of duplicate determinations agreed quite well.

Table V. *Large adductor muscle of* Pecten.

State	Weight g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Total %
		P. opercularis.			
Rest	500	0·016	—	—	—
Phos. max.	500	0·025	—	—	—
Rest	20·0	0·06	2·50	0·05	2·61
Fatigued	20·0	0·14	2·45	0·06	2·65
Rigor	20·0	0·06	2·57	0·23	2·86
Phos. max.	20·0	0·20	2·29	0·08	2·57
Rest	5·00	0·07	3·26	0·06	3·39
Fatigued	5·00	0·08	3·16	0·04	3·28
Rigor	5·00	0·13	1·84	0·08	2·06
Phos. max.	5·00	0·13	1·36	0·10	1·59
		P. maximus.			
Rest	6·8	0·06	0·16	0·01	0·23
Rigor	5·1	0·07	0·17	0·01	0·25
Fatigued	15·8	0·03	—	—	—
Fatigued	7·5	0·06	—	—	—
Rest	20·0	0·024	0·38	0·05	0·45
Fatigued	20·0	0·032	0·40	0·04	0·47
Rigor	20·0	0·028	0·36	0·05	0·44
Phos. max.	20·0	0·031	0·35	0·08	0·46
Rest	50·0	0·044	0·78	0·07	0·89
Fatigued	50·0	0·090	0·76	0·06	0·91
Rigor	50·0	0·094	0·72	0·10	0·91
Phos. max.	50·0	0·076	0·85	0·06	0·98
		P. varius.			
Rest	1·00	0·02	0·78	0·05	0·85
Fatigued	1·00	0·04	0·77	0·04	0·85
Rigor	1·00	0·09	0·58	0·04	0·71
Phos. max.	1·00	0·10	0·72	0·05	0·87
		P. magellanicus.			
Phos. max.	400	0·0012	—	—	—
Phos. max.	2000	0·0013	—	—	—

The heart muscle and foot muscle of *P. maximus* were also investigated. These were both rather small; the hearts of thirty *Pecten* weighed little over a gram. The *Pecten* heart though apparently an active and efficient organ is very thin and light. Only two sets of estimations were made on the heart muscle and only one on the foot muscle. The results along with those obtained on the small adductor muscle are given in Table VI.

As it was thought that the variation in glycogen content of *Pecten* muscle might follow the reproductive changes of the animal, specimens were examined in different stages of their reproductive cycle. This was done in co-operation with Mr C. Amirthalingam who was investigating spawning in *P. opercularis* at Plymouth. The glycogen content of the large adductor muscle from animals

whose gonads were in different conditions was determined. For this purpose the animals were divided into three classes: (1) with spent gonads, (2) with developing gonads, and (3) with full gonads. The results which are given in Table VII are not so definite as had been hoped from a preliminary experiment.

Table VI. Pecten *small adductor muscle.*

State	Weight g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Total %
		P. opercularis.			
Rest	1·50	0·08	0·45	0·08	0·61
Fatigued	1·00	0·14	0·43	0·06	0·63
Phos. max.	1·50	0·24	0·37	0·04	0·65
		P. maximus.			
Rest	6·0	0·08	0·09	0·11	0·28
Phos. max.	6·0	0·19	0·03	0·11	0·33
Rest	5·0	0·07	0·14	0·05	0·26
Rigor	5·0	0·09	0·10	0·07	0·26
Phos. max.	5·0	0·10	0·07	0·06	0·23
Rest	10·0	0·02	0·42	0·05	0·49
Phos. max.	10·0	0·05	0·19	0·06	0·31
Rest	1·00	0·05	0·42	0·07	0·54
Rigor	1·00	0·14	0·38	0·05	0·57
Phos. max.	1·00	0·18	0·34	0·05	0·57
		Heart muscle.			
Fatigued	0·50	0·14	0·62	0·06	0·82
Phos. max.	0·50	0·48	0·30	0·06	0·84
		Foot muscle.			
Rigor	1·00	0·12	0·08	0·03	0·23

Table VII. *Glycogen content (expressed as percentage) of the large adductor muscle of* P. opercularis *at different stages of the reproductive cycle.*

	Condition of gonad			
Date	Spent	Developing	Full	Mean
Jan. 1	0·68	0·84	0·32	0·61
5	0·76	0·88	0·80	0·81
11	1·50	0·42	1·40	1·11

Table VIII. Buccinum *foot muscle.*

State	Weight g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Total %
Rest	10·00	0·01	3·9	0·04	3·95
Rest	3·00	0·04	1·57	0·05	1·66
Fatigued	3·00	0·08	1·36	0·06	1·50
Phos. max.	3·00	0·10	1·27	0·05	1·42

As an example of gastropod muscle, the foot of *Buccinum*, the common whelk, was used. This is a carnivorous animal of moderately active habits. The shell was cut away from the soft parts and the muscular part of the foot was separated from the gut and other viscera; the operculum was removed and the remaining muscle treated in the usual way. It contained large quantities of glycogen even after mincing in alkaline phosphate solution, as can be seen from Table VIII.

Of the three species of cephalopods—*Loligo forbesi*, *Eledone cirrosa* and *Sepia elegans*—available at Plymouth, the first was seldom obtained alive and *Sepia* was seldom in good condition on arrival at the laboratory, but recovered in the aquarium tanks. All the muscle obtained from these animals was quick in action and moderately tough; it was obtained by cutting open the body and removing the viscera. As inositol occurs in cephalopod muscle, it was estimated in some of the samples examined. The muscle was first extracted with ten volumes of alcohol to one of muscle, in order to precipitate inositol and extract the lactic acid and lower carbohydrates. The inositol was extracted from the residue with 70 % acetone and estimated by precipitation with basic lead acetate and then alcohol as described by Needham [1923]. The precipitate of purified inositol was weighed. Glycogen was estimated in the residue from the acetone extraction. The results of these determinations are given in Table IX.

Table IX. *Cephalopod muscle.*

State	Weight g.	Lactic acid %	Glycogen %	Lower carbo-hydrates %	Total %
		Eledone cirrosa (octopus).			
Head					
Fatigued	50·0	0·018	0·11	0·18	0·31
Arms					
Fatigued	100·0	0·025	0·03	0·02	0·08
		Sepia elegans (squid).			
Rest	50·0	0·015	0·024	0·034	0·073
Rigor	50·0	0·021	0·032	0·044	0·097
Phos. max.	50·0	0·029	0·034	0·046	0·109

Inositol in *Sepia elegans*.

State	Weight g.	Inositol %
Rest	50·0	0·07
Rigor	50·0	0·05
Phos. max.	50·0	0·04

Discussion.

While all the molluscan muscles examined produce lactic acid, they show interesting differences in behaviour. The more primitive groups, lamellibranchs and gastropods, contain large amounts of glycogen in their muscles, while the more highly specialised and active cephalopods contain very little glycogen. None of the muscles can produce any great amount of lactic acid, even when large quantities of glycogen are present. This might be expected of the large adductor muscle of *Pecten*, which contracts quickly and rapidly becomes fatigued; on direct stimulation it becomes passive after eight or ten twitches, and Eggleton (in a private communication) says that no phosphagen could be found in this muscle. On the other hand, it contains relatively large amounts of inorganic phosphate. It can be seen that the lactic acid production of the large adductor muscle varies in the different species examined, being greater in the more active *P. opercularis* than in *P. maximus*, and least in the almost

sedentary *P. varius*. *P. opercularis* has small shells, 3 to 5 cm. in diameter, and swims considerable distances without rest, so that its muscles being frequently used are reasonably active. *P. maximus* is larger and heavier, its shells measure 10 to 15 cm. in diameter, and it apparently seldom swims more than a few feet. *P. varius* is less active than *P. maximus* in its habits, being often attached to stones by its byssus. The order of lactic acid production is the same as that of activity in these species, which indicates the close connection between lactic acid production and muscular activity.

The small adductor muscle contracts much more slowly than the large adductor muscle, but it can do much more work before the onset of fatigue. Thus it can yield more lactic acid on stimulation; in this way or by suspension in sodium phosphate solution, there is a much greater conversion of glycogen into lactic acid than occurs in the large adductor muscle.

These two muscles are referred to as the "motor" and "catch" muscles, respectively [Uexküll, 1912]. They are analogous to the skeletal and plain muscles of vertebrates, though it might be noted that the glycogen content of the adductor muscle is much greater than the glycogen content of vertebrate smooth muscle. The small catch muscle was formerly supposed to remain in the contracted state without expenditure of energy, by means of a ratchet or catch mechanism. The chemical evidence, which shows that this muscle can produce more lactic acid than the large adductor muscle, does not support this hypothesis. The glycogen of the small adductor muscle does not vary to the same extent as that of the large adductor muscle, and it is probable that the glycogen content of the latter varies with sexual changes, although insufficient experiments were carried out to demonstrate this. As the glycogen is not converted into lactic acid by the muscle itself, it is probable that some of the glycogen is isolated in separate storage cells or in the conjunctive tissue, so that the enzyme bringing about the conversion to lactic acid is unable to affect it. The histological examination for glycogen is difficult, owing to the indefinite nature of the stains for glycogen. This *Pecten* muscle is possibly less specialised than muscles of higher animals, as is indicated by the presence of so many other substances such as dextrins, glycine, mytilitol, taurine and succinic acid. The muscle is well supplied with blood vessels, so that lactic acid may be rapidly removed in the living animal.

The heart muscle of *Pecten* produces more lactic acid than any other molluscan muscle examined. In this relation, the mollusca differ from vertebrates. As the small heart of *Pecten* is the only muscle which works continuously yet quickly, it might be expected to produce more lactic acid than the other muscles.

The muscle of *Buccinum* appeared to be like the large adductor muscle of *Pecten*, but the cephalopod muscle was quite different from any other muscle examined. Considering the activity of this muscle the lactic acid production was very small, and the changes in glycogen were negligible. The decrease in inositol suggests that this may be utilised by the muscle.

Annelida.

As a specimen of muscle of the annelida, the body wall of the common earthworm, *Lumbricus*, was taken. The worms were collected in October in a garden and were brought to the laboratory in soil. The gut and other viscera were separated from the body wall, which was collected from several animals and treated as previously described. Considering the slowness of contraction of the muscle it was very soon fatigued. The results are set forth in Table X.

Table X. Lumbricus *body muscle.*

State	Weight g.	Lactic acid %	Glycogen %	Lower carbo-hydrates %	Total %
Rest	3·00	0·04	0·54	0·07	0·65
Fatigued	3·00	0·08	0·52	0·12	0·72
Rigor	3·00	0·10	0·24	0·25	0·59
Phos. max.	3·00	0·26	0·21	0·16	0·63

Echinodermata.

Representing the echinodermata, the Sea Cucumber, *Holothuria nigra*, was examined. The muscles attached to the Aristotle's Lantern of *Echinus* were found to be too small for the purpose and troublesome to dissect, so the longitudinal body muscles of *Holothuria* were dissected out and treated in the usual way. This muscle had previously been used by Hill [1926] for experiments on the viscous elastic properties of muscle as it is extremely viscous and although fairly powerful it is very slow in contraction. Table XI shows the results of analyses.

Table XI. Holothuria nigra *longitudinal body muscle.*

State	Weight g.	Lactic acid %	Glycogen %	Lower carbo-hydrates %	Total %
Rest	3·00	0·08	0·09	0·06	0·23
Fatigued	10·00	0·16	0·07	0·04	0·27
Phos. max.	3·00	0·21	0·04	0·04	0·29

Discussion.

The muscles of *Lumbricus* and *Holothuria* are thus like those of the frog in respect of the change of glycogen into lactic acid. The increase in lower carbohydrates in the muscle of the worm in rigor is probably due to hydrolysis of the glycogen by enzymes of the gut. The amount of glycogen contained in the muscle of *Lumbricus* is comparable with that present in frog muscle; that in the muscle of *Holothuria* is much less.

All the invertebrate muscles examined, with the exception of that of cephalopods, in which case the results are inconclusive, are able to convert glycogen into lactic acid, to an extent depending largely on the activity of the muscle. Although the muscles of lamellibranchs and gastropods contain much more

glycogen than they can convert into lactic acid, the cephalopods appear to have very little glycogen in spite of their greater activity. The muscles of annelida, crustacea and echinodermata are much more closely allied to vertebrate muscles than are those of the mollusca. They can all convert glycogen into lactic acid and rely on glycogen alone as a source of lactic acid. While the muscles of invertebrates do not contain creatine or phosphagen (Eggleton, private communication), which may limit lactic acid production, Meyerhof and Lohman [1927] have found an analogous substance in crustacean muscle. This may be responsible for the fairly high lactic acid production of crustacean muscle.

Table XII. *Average lactic acid production in muscles of various animals.*

Phylum and species	Muscle	Average lactic acid production: Fatigue %	Rigor %	Phos. max. %	Reference
Annelida—					
Lumbricus	Body wall	0·08	0·10	0·25	This paper
Arthropoda—					
Homarus vulgaris	Abdomen	—	—	0·24	Ritchie [1927]
	Abdomen	0·10	0·20	0·25	This paper
	Heart	0·20	0·30	0·50	,,
Palinurus vulgaris	Abdomen	0·20	0·25	0·30	,,
	Heart	0·20	0·20	0·30	,,
Leander serratus	Abdomen	0·15	0·17	0·20	,,
Mollusca—					
Pecten opercularis	Large adductor	0·10	0·10	0·15	,,
	Small adductor	0·15	—	0·25	,,
Pecten maximus	Large adductor	0·05	0·05	0·06	,,
	Small adductor	—	0·10	0·15	,,
	Heart	0·15	—	0·50	,,
	Foot	—	0·10	—	,,
Pecten varius	Large adductor	0·04	0·10	0·10	,,
Buccinum	Foot	0·05	—	0·10	,,
Eledone cirrosa	All	—	0·014	—	Henze [1904]
	All	0·02	—	—	This paper
Sepia elegans	All	—	0·02	0·03	,,
Echinodermata—					
Holothuria nigra	Longitudinal	0·15	—	0·20	,,
Vertebratata—					
Melanogrammus aeglifinus (haddock)	Body	—	0·30	—	Ritchie [1927]
Rana temporaria	Skeletal	0·35	0·50	0·80	Meyerhof [1920]
	Heart	—	0·25	—	Arning [1927]
Testudo (tortoise)	Skeletal	—	0·40	—	,, [1927]
	Heart	—	0·30	—	,, [1927]
Felis domestica (cat)	Skeletal	0·20	0·50	0·60	Katz and Long [1925]
	Heart	0·10	0·25	0·30	,, ,, [1925]

Furusawa and Kerridge [1927] have measured the change in acidity of various muscles of marine animals by means of the glass electrode, and found a definite increase on preservation for 24 hours. Assuming that the change in acidity of the muscle on standing is due to lactic acid production, their figures indicate that acid production in invertebrate muscle is of the same order as that in the muscles of vertebrate animals. With

the exception of crustacean heart muscle (*Maia* heart) the results of lactic acid estimation are in agreement with the determinations of acidity. It is possible that crustacean heart muscle contains more efficient buffers than other muscle and so remains relatively alkaline, in spite of acid production. The results of p_H measurements on invertebrate muscle may not represent lactic acid production alone, as autolysis of the protein may affect the acidity of the tissue. Invertebrate tissues autolyse much more quickly than those of vertebrate animals and it is possible that considerable changes in protein may occur in 24 hours. The crustacean heart may produce more basic proteins on hydrolysis; this would account for the discrepancy between the two sets of results.

The lactic acid production under various conditions, of some of the animals examined, is shown in the final table (Table XII).

PART III. VERTEBRATE CARDIAC MUSCLE.

In comparing the lactic acid production of cardiac and skeletal muscle in the frog and tortoise, Arning [1927] found that skeletal muscle gave much more lactic acid in rigor than did cardiac muscle. The relatively low lactic acid production of the heart may be due in part to low buffering power of the tissue. Katz and Long [1925] examined the lactic acid production of mammalian cardiac muscle; they found that the heart was much more dependent on a continuous oxygen supply than is skeletal muscle and that cardiac muscle contained extremely small quantities of glycogen. Thus, though cardiac muscle produces relatively little lactic acid in alkaline phosphate solution, the lactic acid so produced is greater than the amount of glycogen consumed. Katz and Long suggested that this extra lactic acid came from hexosephosphates or lower carbohydrates of the tissue.

Examination of the changes of lactic acid, glycogen and lower carbohydrates in heart muscle showed that on standing in chloroform vapour or in alkaline phosphate solution the amount of lower carbohydrate increased along with the lactic acid. In this heart muscle resembles ordinary skeletal muscle, as lactic acid production involves an increase of hexosephosphate according to the equation advanced by Meyerhof [1924].

$$(C_6H_{10}O_5)_n + nNa_2HPO_4 = nC_6H_{11}O_5PO_4Na_2$$
$$2C_6H_{11}O_5PO_4Na_2 \quad = C_6H_{10}O_4(PO_4Na_2)_2 + 2C_3H_6O_3.$$

The additional lactic acid of heart muscle must therefore come from some other source, in which case its chemical changes during activity differ from those of vertebrate skeletal or invertebrate heart muscle, as the latter has previously been shown (pages 366 and 371) to produce lactic acid in amounts equivalent to the carbohydrates utilised. Determinations of the respiratory quotient of the mammalian heart-lung preparation by Bayliss, Müller and Starling [1928], which were between 0·90 and 0·95, indicate that some non-carbohydrate substance is utilised by the heart under these conditions. This

is in agreement with the low possible oxygen debt observed by Katz and Long, but it is improbable that heart muscle suspended in phosphate solution under strictly anaerobic conditions should be able to convert either fat or protein into lactic acid.

Examination of the constituents of muscle, which could yield this extra lactic acid, showed that inositol might be a possible source, as it occurs to a greater extent in cardiac muscle than in any other vertebrate tissue. The lactic acid which could not be accounted for by carbohydrate amounts to 0·1–0·2 % of the muscle, while the amount of inositol found in mammalian hearts varies from 0·1 to 0·25 % [see Needham, 1926].

The change of inositol into lactic acid is chemically possible; in Abderhalden's *Handbuch der Biochemie* it is stated that electrolysis of inositol produces lactic acid, but no reference is given. A solution of inositol does not readily conduct an electric current; in an attempted electrolysis no lactic acid was formed though it is improbable that optimum conditions for the change were obtained. There is physiological evidence supporting the metabolism of inositol by the heart. Jacobs [1907] found that inositol improved the activity of the perfused frog heart better than addition of glucose. Later, Hewitt and De Souza [1921] observed that small amounts of inositol increased the amplitude of a perfused heart either in the normal condition or treated with atropine.

Some preliminary experiments in which inositol was estimated in heart muscle showed that the amount present diminished under conditions which usually lead to lactic acid formation. Further experiments in which inositol was added to minced cardiac muscle showed that the addition increased the amount of lactic acid formed. An attempt to extract an enzyme from cardiac muscle which would convert inositol into lactic acid was, however, unsuccessful.

Experimental.

The heart, removed from a decerebrate or dead animal as rapidly as possible, was finely minced and samples were taken for treatment and analysis by methods described in a previous communication [1928]. The pig heart was obtained from the slaughter-house immediately after the animal had been killed, and experiments were usually started about half an hour after the death of the animal.

The first experiments on tortoise muscle show the increase in lower carbohydrates of both cardiac and skeletal muscle when allowed to produce lactic acid in phosphate solution. The relatively great variation of tortoise skeletal muscle which had previously been noted by Arning tends to lessen the value of the comparison of the muscles. The results are recorded in Table I.

In order to follow the change of inositol large samples of a minced heart taken from a pig were treated and inositol was estimated, in addition to the other substances concerned. Inositol was estimated by a modification of the method described by Needham [1923]. The modification consisted in a direct weighing of the inositol, in place of a micro-estimation of carbon. The method is probably

not very specific, but a more reliable method could not be found. The incubation was always carried out in the presence of chloroform in order to prevent bacterial action. Two sets of experiments made on two different samples are shown in Table II. In the experiment in which inositol was estimated, it was utilised to an extent that almost accounts for the excess lactic acid.

Table I. *Tortoise muscle.*

State	Weight g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Total %
		Heart.			
Normal	1·19	0·24	0·32	0·09	0·65
Normal	1·52	0·31	0·37	0·04	0·72
Normal	1·84	0·22	0·41	0·05	0·68
Phos. max.	0·63	0·49	0·10	0·15	0·74
Phos. max.	1·54	0·51	0·13	0·18	0·82
Phos. max.	1·52	0·61	0·11	0·12	0·84
		Skeletal.			
Normal	10·0	0·12	0·52	0·15	0·79
Normal	10·0	0·09	0·75	0·16	1·00
Phos. max.	10·0	0·68	0·06	0·23	0·97
Phos. max.	10·0	0·79	0·02	0·23	1·04

Table II. *Lactic acid, glycogen, lower carbohydrates and inositol in pig heart.*

State of muscle	Weight g.	Lactic acid %	Glycogen %	Lower carbohydrates %	Inositol %	Total %
Normal	50·0	0·19	0·21	0·06	0·37	0·83
Incubated 6 hrs. at 37° with $CHCl_3$	50·0	0·30	0·15	0·08	0·33	0·86
Incubated 24 hrs. at 37° with Na_2HPO_4	50·0	0·35	0·17	0·15	0·25	0·92
Normal	50·0	0·09	0·06	0·05	Not estd.	0·20
Incubated with Na_2HPO_4	50·0	0·16	0·03	0·08	„	0·27

The effect of adding known amounts of inositol (B. D. H.) to incubating minced heart muscle was next tried. The lactic acid produced was compared with that produced in a control experiment. When the muscle was shaken in a thermostat (Table III, Exp. 1) there was an increase in lactic acid production when inositol was added, but in Exp. 2, in which the muscle was allowed to stand in the incubator, no increase in lactic acid was detected.

Table III. *The effect of inositol on lactic acid production of pig heart muscle in 20 hours at 37°, in presence of* Na_2HPO_4 *and excess of toluene.*

	Lactic acid produced mg.	
Exp. 1.		
10·0 g. muscle	17·0	with stirring
10·0 g. muscle with 5 mg. KCN	17·8	with stirring
10·0 g. muscle with 50 mg. inositol	32·8	with stirring
10·0 g. muscle with 50 mg. inositol and 5 mg. KCN	27·6	with stirring
Exp. 2.		
50·0 g. muscle	93	
50·0 g. muscle with 100 mg. inositol	88	

Discussion.

The results obtained on cardiac muscle, which are incomplete, are published at this stage because the work cannot be continued at present. They show that the extra lactic acid production of mammalian cardiac muscle is not accompanied by a decrease in lower carbohydrates, on the contrary, the lower carbohydrates of muscle have a tendency to increase in amount when lactic acid is produced. On the other hand, in one experiment lactic acid production was coupled with a decrease in inositol and in another experiment the addition of inositol to incubating heart muscle increased the lactic acid production, though the whole of the inositol did not appear to be utilised.

Summary.

1. The change of glycogen into lactic acid has been found to accompany activity in invertebrate muscle in the same way as in the muscle of vertebrate animals.

2. The quantity of lactic acid produced under various conditions is different for different species.

3. The heart muscle of invertebrates differs from that of vertebrate animals in not producing less lactic acid than the skeletal muscle of the same animal and in not producing lactic acid in excess of the glycogen present.

4. The muscles of crustacea, lamellibranchs and gastropods appear to act as stores for glycogen.

5. Muscles of lamellibranchs and gastropods usually contain large quantities of glycogen which cannot be converted into lactic acid by the muscle under known optimal conditions for this change.

6. Vertebrate cardiac muscle produces more lactic acid than can be accounted for by the consumption of carbohydrate. It is suggested that this excess lactic acid is derived from the inositol present in cardiac muscle.

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XLIX. PRELIMINARY INVESTIGATIONS ON THE CONSTITUTION OF THE HEMICELLULOSES OF TIMBER.

By MARGARET HELENA O'DWYER.

From the Chemistry Department, United College of St Salvator and St Leonard, University of St Andrews.

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Introduction.

Following on the work described in a previous communication [O'Dwyer, 1926], an attempt has been made in the present investigation to obtain further information regarding the chemical constitution of the hemicelluloses of timber, and thereby, if possible, to gain a better knowledge of their function in plant life.

The first section of the work has been carried out on a small quantity of the so-called *hemicellulose A* from beech wood, the preparation and hydrolysis products of which have already been described [O'Dwyer, 1926]. This substance is obtained by treating sawdust in the cold with 4 % sodium hydroxide, after the removal of the water-soluble constituents, the pectic substances and the protein. Hemicellulose A bears very little resemblance to cellulose in physical properties or, so far as can be judged, in chemical constitution.

The general method of investigation employed was to subject the hemicellulose to mild acetylating reagents which would be unlikely to cause profound depolymerisation on hydrolysis. In this way it was expected that a uniform acetate would be obtained, which, owing to its solubilities, could be purified from extraneous compounds and permit of more accurate study. Some idea of the complex nature of the investigation may be gathered from the fact that the acetate prepared from this material can be separated into three different fractions, and that whereas 80 % of the hemicellulose from beech wood, already referred to, could be readily acetylated, only 20 % of hemicellulose A from an older beech and from oak heartwood responded to the same treatment. This resistance to acetylation is not attributable to the accumulation of inorganic constituents in the fraction of the hemicellulose unaffected by the reagents and must be regarded as a specific property. Even the application of the more drastic acetylating mixture used in the acetolysis of cellulose [Irvine and Robertson, 1926] had little effect and the conclusion might have

been drawn that no hydroxyl groups were present. That such is not the case was shown by subjecting the material which could not be acetylated to the effect of methyl sulphate and alkali. Normal methylation then took place until the value OMe = 15·7 % had been obtained, but thereafter a sodium derivative was produced and this interfered with carrying the alkylation to its extreme limit.

A detailed examination of hemicellulose B has not been possible at this stage owing to the low percentage of that substance obtained from oak heartwood, due, probably, to the age of the wood. In the course of the work it was found that hemicellulose, even when purified exhaustively from pectin, contains methoxyl and reference may now be made to this unexpected observation.

A series of experiments showed that about 50 % of the methoxyl present in hemicellulose A could be readily de-esterified, but the remainder proved absolutely resistant even when drastic methods of hydrolysis were employed. It is, therefore, reasonable to conclude that methoxyl is present in two forms, both as a methyl ester and as a methoxy-derivative. The occurrence of methoxyl in the hemicelluloses can hardly be ascribed to the pectic substances present in wood, for these substances are found in very small quantities in lignified tissue and any which had not been removed by previous treatment of the sawdust [O'Dwyer, 1926] would be de-esterified by the 4 % sodium hydroxide used for the extraction of the hemicellulose. In addition, as pectic substances are soluble in water they are unlikely to be present as an impurity in hemicellulose A.

The possibility that lignin may be the source of the methoxyl detected in hemicellulose has not been overlooked. Mehta [1925], and Dorée and Barton-Wright [1927] found that a soluble form of lignin (termed by the latter authors "metalignin") could be isolated in relatively large amount by extracting wood sawdust for 1 hour with 4 % sodium hydroxide under a pressure of from 8 to 10 atmospheres. Further, Hawley and Campbell [1927] record that, after the extraction of Sitka spruce sawdust with 1 % sodium hydroxide for 1 hour on a boiling water-bath, the lignin content of the wood had fallen from 29·3 % to 27·7 %.

The hemicelluloses described in the present paper were obtained by extracting sawdust in the cold with 4 % sodium hydroxide for 48 hours, the mixture being stirred mechanically, and it is conceivable that not only would some metalignin pass into solution during this treatment but that it would be precipitated with the hemicellulose on the subsequent addition of acetic acid, despite the fact that metalignin is soluble in that reagent. Metalignin is, however, likewise soluble in alcohol and as the precipitated hemicelluloses are purified by immersion in alcohol of gradually increasing concentrations for a period of from two to three weeks and finally washed several times on the pump with absolute alcohol before being dried, it seems unlikely that any considerable amount of metalignin would be present in the final product.

As the methoxyl content of metalignin is 17·4 % [Dorée and Barton-Wright, 1927] 24 % of this compound must be assumed to be present in the hemicellulose A examined, if the methoxyl is to be attributed to the former alone. The history of the material makes this supposition unlikely, and it seems evident therefore, that little, or none, of the methoxyl in hemicellulose can be ascribed to the presence of lignin.

The initial work on beech hemicellulose was of value in suggesting methods for the more detailed investigations which were later carried out on the corresponding product from oak. Unfortunately even in this second instance the work has been hampered by insufficient quantities of material and for this reason has had to be suspended, for the moment, at an interesting stage.

Although this investigation has, so far, not produced sufficient evidence on which to assign a chemical constitution to the hemicelluloses it has nevertheless yielded valuable results and has afforded further evidence that, as stated in the previous communication, these substances are apparently more akin to pectin than to cellulose and that they may even bear some slight resemblance to lignin.

Von Fellenberg [1918] and Ehrlich [1927] have already suggested the possibility of a transition in the plant from the pectic substances to lignin. If such be the case, it is conceivable that the hemicelluloses may prove to be transition products in this important natural transformation. As has been previously pointed out [O'Dwyer, 1926], such unlignified tissue as has been examined contains a much larger proportion of pectic substances than of hemicellulose, whereas experiments conducted on lignified tissue have shown the opposite to be the case. Again, in unlignified tissue, hemicellulose B occurs in larger quantities than hemicellulose A, and in lignified tissue the reverse holds true. The timber examined so far has shown that the percentage of hemicellulose B tends to diminish with the age of the wood, and a point might conceivably be reached at which this substance would disappear altogether. Hemicellulose A might also become much modified as lignification proceeds further.

Experimental.

Acetylation of beech hemicellulose. The first set of experiments was carried out on a small quantity of hemicellulose A from beech wood 80 years old, containing 3·7 % methoxyl and 2·7 % ash. The hydrolysis products obtained from this substance have already been examined [O'Dwyer, 1926].

An endeavour was made to obtain an acetate by the method of Barnett [1921] using certain modifications suggested by Irvine and Hirst [1922]. Several experiments were carried out with small quantities of hemicellulose and in each case a white, amorphous product was obtained which was not completely soluble in either chloroform or pyridine and was insoluble in other organic solvents. Fractional precipitation of the chloroform solution with

alcohol gave a second substance soluble in both chloroform and pyridine. Table I shows the results obtained.

Table I.

Experiment	Quantity of hemicellulose taken g.	Temperature and time of experiment	Amount of acetate obtained g.	Acetyl groups before fractionation %	*Specific rotation* Fraction I	Fraction II
1.	5	65–70° 36 hours	5·5	49·5	$[\alpha]_D$ −36° (pyridine) (cloudy) c=1·774	$[\alpha]_D$ −52° (pyridine) (clear) c=2
2.	5	65–70° 75 hours	5·4	49·0	$[\alpha]_D$ −19° (chloroform) c=1·5 $[\alpha_D]$ −20° (pyridine) c=1·8 (cloudy)	$[\alpha]_D$ +28° (chloroform) c=2·03 $[\alpha]_D$ +20° (pyridine) c=2·00 (clear)
3.	5	45–50° 36 hours	3·4	48·2	$[\alpha]_D$ −52° (chloroform) (cloudy) c=0·9520	$[\alpha]_D$ −60° (chloroform) (clear) c=1·070 $[\alpha]_D$ −55° (pyridine) (clear) c=0·9300
4. Residue from 3.	1·6	45–50° 36 hours	1·5	45·04	$[\alpha]_D$ −54° (pyridine) (clear) c=1 $[\alpha]_D$ −60° (chloroform) (clear) c=1·02	$[\alpha]_D$ −10° (chloroform) (clear) c=0·992 $[\alpha]_D$ −3·8° (pyridine) (clear) c=1·634

From these results it seems evident that the acetates contain at least two and possibly three fractions and that prolonged acetylation at a temperature of 65–70° results in de-polymerisation of the substance. A sixth acetylation was carried out on 8·3 g. of hemicellulose A from an older beech wood (130 years) at 40° for 3 days. Only 1·1 g. of acetate was obtained and no further yield was possible by treating the hemicellulose at 50° and at 65–70°. This substance therefore shows marked differences as regards acetylation compared with hemicellulose A from younger beech.

Hydrolysis of acetate from beech with acid methyl alcohol. 2·4706 g. containing both fractions of the acetate obtained at 45–50° were treated in a sealed tube at 100–105° by the method of Irvine and Hirst [1922] with 30 cc. pure methyl alcohol containing 0·5 % dry hydrogen chloride. At the end of 115 hours 14 % of the substance still remained undissolved. This residue consisted of a greyish amorphous powder, volatile on ignition, free from nitrogen, sulphur and carbohydrates. The ash content was negligible. It contained 2·4 % methoxyl.

After filtering off the residue, the acid methyl alcohol solution was subjected to further treatment by Irvine and Hirst's method. 1·0346 g. of uncrystallisable syrup was obtained: $[\alpha]_D$ (in acid methyl alcohol) + 92°, c = 2·06. The

syrup was then extracted with ether and 0·1412 g. of a substance, soluble in that reagent but insoluble in water, was obtained. It had $[\alpha]_D$ (in alcohol) + 26°, $c = 0{\cdot}5624$. As the residue insoluble in ether still failed to crystallise, the methoxyl content was determined and 19·9 % OMe obtained.

Hydrolysis of the syrup with hydrochloric acid. For this purpose 0·4709 g. was treated with 25 cc. 3 % hydrochloric acid [Irvine and Hirst, 1924]. The syrup was not completely soluble in the acid, so that a cloudy solution resulted of which the rotation could not be taken. After heating at 100° for 30 minutes a brown precipitate separated. This was filtered off and the hydrolysis continued. The residue weighed 0·0378 g. It melted at 166°.

The following polarimetric results were obtained during the progress of the hydrolysis.

Table II.

Time of hydrolysis ($c = 1{\cdot}852$)	30 mins.	75 mins.	120 mins.	165 mins.	200 mins.	245 mins.
α	+0·55°	+0·42°	+0·25°	+0·18°	+0·15°	+0·15°
$[\alpha]_D$	+60°	+45·8°	+27·3°	+21·4°	+18·4°	+17·8°

The last three sets of figures were calculated on the weight of the sugar formed during hydrolysis [*vide* Irvine and Hirst, 1922]. The hydrolysed solution was neutralised, taken to dryness under reduced pressure, and the residue dissolved in hot absolute alcohol. Weight of sugar formed 0·9460 g. M.P. 146°. $[\alpha]_D$ +18° (in water), $c = 3{\cdot}65$. The sugar is therefore xylose.

Hemicellulose A (Oak).

Such information as had been gained by the study of hemicellulose A from beech was now applied to 60 g. of the same substance from American oak heartwood, the age of which is not definitely known. This substance was prepared in a similar manner to the beech hemicellulose, and contained 4·8 % OMe, 2·4 % ash (mostly silica) and a very small quantity of phosphorus.

(*a*) *Extraction of oak hemicellulose A with alcohol.* Two extractions with boiling absolute alcohol were carried out in order to effect further purification of the substance. On taking the alcoholic extract to dryness a small amount of residue was obtained having the odour of vanillin, insoluble in water, M.P. 162° (approx.) and containing 5 % OMe. The hemicellulose after the alcoholic extraction still contained 4·1 % OMe.

(*b*) *Examination of methoxyl groups in oak hemicellulose A.* In order to ascertain whether any of the methoxyl was present in the form of an ester, a series of experiments was carried out on small quantities of the hemicellulose after purification. Amounts varying from 2 to 3 g. were treated with 0·5 *N* sodium hydroxide at temperatures ranging from 65° to 85° for 2 hours. The hemicellulose was recovered as a white powder, after dialysis of the solution with distilled water, by the addition of acetic acid and alcohol, and was dried in the usual way [O'Dwyer, 1926]. In each case a methoxyl content of about 2 % was obtained for this substance. It is evident, therefore, that about 50 % of the methoxyl present in the hemicellulose resists de-esterification and

is present in the substance in a very stable form. Some additional experiments on the hemicellulose with different strengths of ammonia showed that the methoxyl content again dropped to 2 % after the substance had stood for some time in the cold with 10 *N* ammonium hydroxide, but that 4 *N* ammonium hydroxide under similar conditions had apparently no de-esterifying effect.

(*c*) *Acetylation of oak hemicellulose A.* Barnett's method was employed with modifications as in the case of beech hemicellulose. The first estimation was carried out at 40–45° for 36 hours, as these conditions apparently gave the best results in the acetylation of beech hemicellulose. As, however, the yield of acetate from this experiment was only a little over 10 %, the time was extended to 100 hours in a second experiment. The amount of acetate obtained, however, was only about 20 %. Raising the temperature in subsequent experiments to 80° produced no more acetate, and it became evident that the acetylation could be carried no further.

Under these circumstances a more drastic method was employed [Irvine and Robertson, 1926]. 1 g. of the hemicellulose which remained as a residue after acetylation was carefully added to a mixture consisting of 1 cc. sulphuric acid and 10 cc. acetic anhydride. The resulting mixture was kept at 0° for 1 hour to prevent charring, and then at room temperature for 3 days. It was next plunged into a large volume of water, the precipitate filtered off and dried. It weighed 0·79 g. and proved to be unchanged hemicellulose. A very small quantity of acetate was obtained. The experiment was repeated with 5 g. of hemicellulose residue.

Yield of acetate	0·1 g. (grey in colour)
Residue (unchanged hemicellulose)	4·69 g.
OMe in residue	2·16 %
Ash in residue	2·9 %

Table III shows the results obtained from the first series of acetylations.

(*d*) *Hydrolysis of acetate from oak with acid methyl alcohol.* The fractions of the acetate soluble in chloroform were combined and treated with acid methyl alcohol in the same manner and under the same conditions as in the case of beech acetate.

Amount of substance taken	1·5673 g.
,, syrup obtained	0·5692 g.
,, residue obtained	0·0978 g.

On treatment of the syrup with hot absolute alcohol fine white crystals were obtained. Wt. 0·2375 g., M.P. 150°, $[\alpha]_D$ (in alcohol) $-62°$, $c = 2$. These crystals were apparently those of β-methylxyloside. Owing to the small yield it was impossible to recrystallise.

(*e*) *Methylation of unacetylated hemicellulose.* As the acetylation of this substance could be carried no further, it was decided to attempt the methylation of the unacetylated residue by dimethyl sulphate and sodium hydroxide. For this purpose a small quantity of hemicellulose, which had been submitted to acetylation by both processes, was employed. 3 g. were dissolved in 30 cc.

Table III.

Experiment	Quantity of substance taken g.	Temp. and time of experiment	Amount of acetate obtained g.	Acetyl groups in combined fractions %	Specific rotation of Fraction II. (Fraction I is insoluble in organic solvents)
I (hemicellulose)	5	40–45° 36 hours	0·6	43·0	$[\alpha]_D - 75°$ (pyridine) (cloudy) $c = 2$
I (*a*) Residue from I	4·2	60° 30 hours	0·9	43·9	$[\alpha]_D - 74°$ (pyridine) (cloudy) $c = 0·67$
II (hemicellulose)	10	40–45° 100 hours	2·06	42·5	$[\alpha]_D - 76°$ (pyridine) (very cloudy) $c = 2$ (approx.)
II (*a*). Acetate from II	1·6	70° 10 hours (Slight discoloration of substance)	0·8	49·0	$[\alpha]_D - 43°$ (chloroform) (cloudy) $c = 2·012$ $[\alpha]_D - 28°$ (pyridine) (less cloudy) $c = 2·0$
III (hemicellulose)	10	60–65° 36 hours	1·85	42·0	$[\alpha]_D - 72°$ (pyridine) (cloudy) $c = 1·2$
IV (hemicellulose)	10	70–75° 36 hours	2·3	44·8 After fractionation: Frac. I. 43·0, Frac. II 50·0	$[\alpha]_D - 76°$ (chloroform) (cloudy) $c = 0·1938$

				After fractionation		Specific rotation after further fractionation	
				Frac. I	Frac. II	Frac. II *a*	Frac. II *b*
V (hemicellulose)	14	65–70° 36 hours	4·8 Separated into two fractions by chloroform	43·1 Insol. in org. solvents	50·0 Sol. in chloroform	$[\alpha]_D - 64°$ (chloroform) (clear) $c = 2·075$	$[\alpha]_D - 35·6°$ (chloroform) (clear) $c = 2·083$

The effect of acetylation on the acetate soluble in chloroform seems to be de-polymerisation, unless—as suggested by the higher acetyl value of Fraction II in the above experiment—a further separation might be effected and a third substance obtained. The proportion of material insoluble in chloroform in these acetates is much greater than in those obtained from beech.

of 2 *N* sodium hydroxide. The temperature was then raised to 37° and 27 cc. of 30 % sodium hydroxide and 8 cc. of dimethyl sulphate were run in slowly with stirring, the temperature during this process being kept at 37°. It was then raised to 100° for 30 minutes after which the mixture was cooled, acidified with hydrochloric acid and made faintly alkaline with solid sodium carbonate. It was then taken to dryness, extracted with boiling chloroform and the liquid filtered off and evaporated to dryness. The amount of the substance soluble in chloroform or in alcohol was negligible. In order to free it from inorganic matter the mixture was dialysed for several days and a precipitate obtained from the colloidal solution remaining in the dialyser by the addition of acetic

acid and alcohol. This precipitate was dried in a similar manner to that employed in the case of hemicellulose. It was a fine white powder (1·8480 g.) having the same chemical properties as hemicellulose A and containing 13·7 % OMe and 1·2 % ash.

As the methoxyl content of the substance had been raised to a considerable extent by the action of dimethyl sulphate and sodium hydroxide, 20 g. were methylated under the same conditions. The resulting product was similar to that obtained in the last experiment. It weighed 13 g., contained 15·7 % OMe, and 2·2 % ash which was identified as silica.

10 g. of the substance obtained from the last experiment were now submitted to a second methylation under the same conditions as in the first experiment. On taking the chloroform extract to dryness a very small amount of substance, 0·2790 g., was found to be soluble in that reagent. This product was obtained, not as a syrup but as an amorphous, fine, yellow powder, giving a methoxyl content of 30·2 %. The remainder of the material obtained as a result of the methylation was dialysed as before and a precipitate obtained by the addition of acetic acid and alcohol. A considerable loss occurred during the methylation process as only 3·5 g. of a greyish powder were obtained. This was sparingly soluble in sodium hydroxide and much less hygroscopic than that obtained after the first methylation. It contained 14·76 % ash. The methoxyl content of the ash-free substance was 27·8 %. An attempt to determine the rotation in 2 % sodium hydroxide showed that the substance was slightly laevorotatory, but an exact figure could not be obtained.

Table IV.

	Substance	Percentages of C	H	OMe	Ash	Specific rotation	Soluble in	Insoluble in
a.	Hemicellulose A	44·68 44·56	6·51 6·43	4·1	1·85	$[\alpha]_D$ −125° (in 2 % NaOH) *c* = 0·240	Dilute alkali	Dilute acids, water and organic solvents
b.	Acetate from hemicellulose A — fraction I	41·18 40·99	6·32 5·98	nil	1·28	Unobtainable		All organic solvents and acetic acid and cold alkali
c.	Acetate from hemicellulose A — fraction II	46·38 46·52	6·37 6·05	nil	negligible	$[\alpha]_D$ −75° (in chloroform) *c* = 2	Chloroform and pyridine	Water, alcohol and most other organic solvents
d.	Residue hemicellulose A after acetylation	44·12 43·88	5·90 5·91	2·2	1·52	$[\alpha]_D$ −75° (in 2 % NaOH) *c* = 0·20	Similar to hemicellulose A	
e.	Product obtained from methylation of (*d*)	43·71 43·70	6·56 6·83	15·7	2·26	$[\alpha]_D$ −101° (in 2 % NaOH) *c* = 0·2167	Similar to hemicellulose A	
f.	Product obtained from methylation of (*e*)	42·94 43·23	7·04 7·25	27·8	14·76	Small laevorotation, exact figure unobtainable	Sparingly in dilute alkali otherwise solubilities similar to hemicellulose A	
g.	Product obtained after attempted acetylation of (*f*)	47·57 47·75	7·26 7·37	28·2	5·4	$[\alpha]_D$ −5·2° (in chloroform) *c* = 1·632	Chloroform	Alcohol and water

The carbon content of (*f*) is obscured by the high percentage of ash. All results were calculated on an ash-free basis.

The high percentage of ash—a rise of about 12·5 % on that obtained after the first methylation—suggests the formation of a sodium compound during methylation. A substance, for instance, having the composition $C_6H_9O_5Na$ would contain 12·5 % sodium and this figure corresponds almost exactly to the increase noted in the ash.

(*f*) *Attempted acetylation of the methylated product.* An attempt was made to acetylate the product obtained after a second methylation by Barnett's method. This was not successful, but after neutralising the filtrate from the acetylation with barium carbonate, filtering, evaporating the filtrate to dryness and extracting the residue with boiling chloroform 0·7182 g. of a substance soluble in that reagent, and insoluble in water and alcohol, was obtained.

Table IV gives the ultimate analysis, methoxyl content, percentage of ash, specific rotation and solubilities of the substances obtained during the investigations on oak hemicellulose.

Hemicellulose B (Oak).

About 5 g. of this product was obtained from 2 kg. of the oak heartwood. It was similar in character to that found in beech and described in a previous communication [O'Dwyer, 1926].

The following results were obtained from this substance after extraction, as in the case of hemicellulose A, with boiling absolute alcohol.

Percentages of				
C	H	OMe	Ash	*Specific rotation*
37·27	6·67	3·4	5·8	$[\alpha]_D$ −66° (in 2 % NaOH) c=0·2100
37·34	6·37			

These results were calculated on an ash-free basis.

Summary.

Preliminary investigations into the constitution of the hemicelluloses of timber have been carried out on hemicellulose A from beech wood and from American white oak.

Some idea of the complex nature of these investigations may be gathered from the fact that the acetates obtained contain apparently three different substances.

80 % of hemicellulose A from beech wood 80 years old could be readily acetylated, but only 20 % of the same substance from an older beech and from oak heartwood responded to the same treatment. Hence the nature of the hemicelluloses depends, apparently, on the age of the wood.

The hemicelluloses from both beech and oak contain a certain percentage of methoxyl of which about half exists in the form of an ester. The remainder seems to bear a different character as it resists drastic methods of de-esterification. The determination of the source of the methoxyl presents interesting possibilities.

Methylations have been carried out on the residue from acetylation in the case of oak, and a considerable rise in the methoxyl content has been noted, thereby confirming the presence of unsubstituted hydroxyl groups. This, however, was accompanied by a large increase in ash after re-methylation of the substance, due to the formation of a sodium compound.

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L. THE BIOLOGICAL SIGNIFICANCE OF THE UNSAPONIFIABLE MATTER OF OILS.

IV. THE ABSORPTION OF HIGHER ALCOHOLS.

By HAROLD JOHN CHANNON and
GEORGE ARTHUR COLLINSON.

From the Department of Experimental Pathology and Cancer Research, University of Leeds.

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In a previous paper [Channon, 1926], it was reported that the unsaturated hydrocarbon squalene is absorbed from the alimentary tract by the rat. Its absorption caused a large increase in the amount of the unsaponifiable fraction of the liver and its presence could be demonstrated in that fraction. It was suggested that the large increase in the amount of the liver cholesterol which occurred at the same time might be due to some chemical relationship existing between squalene and cholesterol. Alternatively, that increase might be due to the fact that, as the amount of the unsaponifiable fraction had increased on account of the absorption of squalene, the amount of cholesterol had also increased in order that the ratio of cholesterol to other unsaponifiable substances present might remain as constant as possible. The work described in this paper was carried out with a view to throwing further light on these two hypotheses.

Brief reference may be made to the literature concerning the absorption of alcohols and hydrocarbons. Munk and Rosenstein [1891] administered cetyl palmitate to a patient with a lymph fistula and found that the chyle which was subsequently collected for 14 hours contained neither cetyl palmitate nor cetyl alcohol, but tripalmitin only. Further experiments with amyl oleate yielded results which confirmed the previous finding that hydrolysis of esters precedes absorption of the fatty acids. Similar results were obtained by Frank [1898], who administered the ethyl esters of the higher fatty acids and found only the glyceryl esters in the chyle. More recently, Bloor [1912] sought to overcome the difficulty of detecting small quantities of such substances in the chyle by administering to dogs with thoracic fistulae "isomannide" esters of high optical activity. He found, however, no trace of optically active substance in the chyle. The results of these workers make it appear established that the esters of higher alcohols are first hydrolysed before the absorption of their constituent fatty acids. A number of experiments have been carried

out in which the absorption of unsaponifiable substances themselves have been studied. Connstein [1899] using the intake and excretion method found that almost all the 20 g. of lanoline which he administered to a dog was excreted in the faeces, and Henriques and Hansen [1900] carried out similar experiments with a mixture of equal parts of lard and vaseline and found that 95 % of the vaseline was excreted. Bloor [1913] administered a liquid hydrocarbon mixture and also vaseline, alone and in an emulsion in olive and coconut oils, and recovered in the faeces 85·5 to 100 % of the hydrocarbons. He also carried out experiments on animals with thoracic fistulae using similar hydrocarbon mixtures. The unsaponifiable fraction of the chyle fat was prepared and in every case it proved to be a solid of M.P. 110° (cholesterol, M.P. 148·5°). The only workers who claim that absorption of a hydrocarbon occurs are Bradley and Gasser [1911], who administered an emulsion of olive oil and petroleum to a dog and found that the thoracic lymph obtained by fistula contained both the oil and the hydrocarbon in about the same relative proportions as in the emulsion which was administered; they conclude that "this suggests a mechanical absorption of droplets of fatty acids and hydrocarbon oil mixtures."

In the present investigation use has been made of the difference method in which animals in two groups are fed on a given diet and to one group the unsaponifiable substance is given as a small daily supplement. The unsaponifiable matter is prepared from the faeces of the two groups of animals collected over the period of experiment, and the difference between these weights of unsaponifiable material taken as representing the amount of the unsaponifiable substance excreted. Not too great a reliance may be placed on such a figure, however, firstly because the collection of the faeces is difficult and it is impossible to be sure that some loss of material has not occurred, and secondly because the output of unsaponifiable substances in the faeces varies somewhat even among carefully chosen groups of animals on a standard diet. In this connection, reference may be made to the work of Sperrey [1927] who found that an animal on a fat-free diet excretes an appreciable amount of unsaponifiable substance in the faeces and that this excretion persists even when the bile is drawn off by means of a fistula. Hence, although every care may be taken to ensure that control animals of similar ages and weights are used, variations in the excretion of unsaponifiable material may occur. It is clearly desirable also to commence and terminate the experiment by the inclusion of charcoal or bone ash to ensure that the collection of the faeces be accurately made. Within the limitation that the difference in the amount of substance administered and the amount excreted must be considerable before any conclusions as to whether absorption occurs can be drawn, this method will serve.

It will be remembered that the unsaturated fatty acids are more readily absorbed than the saturated, probably because they are more readily emulsified but possibly by virtue of their chemical properties. The alcohols used in these experiments—cholesterol, phytol, cetyl and oleyl alcohols—were chosen

with a view to seeing whether chemical structure as opposed to physical properties were a factor involved in the absorption. Although it is known that cholesterol (M.P. 148·5°) is absorbed to some extent, that alcohol was used because the general distribution of sterols in tissues necessitates the daily ingestion of a definite quantity of those substances and, secondly, cholesterol can be accurately estimated by the digitonin method. Phytol, $C_{20}H_{39}OH$ (B.P. 202°, 10 mm.), which is present in all plant tissues containing chlorophyll must likewise be ingested in small amounts. Cetyl alcohol, $C_{16}H_{33}OH$ (M.P. 51°), previously used as the palmitate by Munk and Rosenstein [1891] and the unsaturated oleyl alcohol, $C_{18}H_{33}OH$ (B.P. 207° 13 mm.), are natural products. The results obtained with squalene [Channon, 1926] suggested that it was desirable to determine firstly, whether these alcohols were absorbed and if the absorption could be related to their chemical or physical properties, and secondly, whether their absorption caused an increase in the amount of the unsaponifiable fraction of the liver, and if so, to determine whether there was an increase at the same time in the amount of the liver cholesterol.

EXPERIMENTAL.

Two groups of rats were fed on a complete artificial diet, one of the groups receiving, in addition, a small quantity of the particular alcohol daily. The diet used was that described by Drummond and Coward [1920] with the replacement of the vegetable oil by butter, and the groups of animals were chosen as alike as possible in age, weight and sex. The daily supplements of alcohol were given prior to the food. Oleyl alcohol and phytol were given as drops direct into the animal's mouth. Cetyl alcohol and cholesterol were dispersed in olive oil and given in this way, the control animals in these experiments receiving similar amounts of olive oil daily. In order to facilitate absorption, it was considered desirable to give small doses for some time rather than to administer larger doses over shorter periods.

Other experiments were carried out with cetyl alcohol and cholesterol in which the animals received a diet containing 1 % of these substances. Throughout the experiments the faeces were collected daily and stored in alcohol until the end of each experiment.

Treatment of livers. At the end of the experiment the animals were killed by chloroform and the combined livers of each group were weighed, heated with 5 % potassium hydroxide until solution was effected, and the hydrolysed product was extracted six or seven times with ether in a separating funnel after adding sufficient alcohol to ensure rapid separation of the two layers. The combined ether extracts of each batch of livers were washed with water to remove soap and alcohol, evaporated to dryness and finally dried *in vacuo* at 100°. The material so obtained was then resaponified with sodium ethoxide dissolved in alcohol, and the solution poured into water and extracted with ether as before. The combined ether extracts were washed with water, great care being taken at this stage to ensure by many washings that no soap was

left behind in the ether extract. The ether extract was dried *in vacuo* at 100° and the weight of the unsaponifiable matter obtained. Cholesterol was estimated in an aliquot part by the digitonin method.

Treatment of faeces. The faeces, which had been stored in alcohol, were ground up in the alcohol and filtered; two more grindings with absolute alcohol were made and the residue sucked as dry as possible. The residue was then extracted with ether in a Soxhlet apparatus. All the alcohol washings and ether extracts were combined, and the solvents removed. Two saponifications followed by ether extractions were carried out, as already described for the livers.

Results.

Oleyl alcohol. The oleyl alcohol used in these experiments was prepared by the reduction of ethyl oleate by the method of Bouveault and Blanc [1904]. In both experiments it was administered before the daily food in drops.

Exp. 1	No. of rats	Time days	Weight of livers g.	Weight of unsap. matter g.	Weight of cholesterol g.	Unsap. matter of faeces g.	Oleyl alcohol administered cc.
Control diet	6	30	56	0·2174	0·1880	1·8044	—
Control diet + oleyl alcohol	6	30	60	0·3052	0·2200	3·4416	11·23
Exp. 2							
Control diet	6	15	41·8	0·2195	0·1161	1·2960	—
Control diet + oleyl alcohol	6	15	45·8	0·2636	0·1711	1·5918	5·26

Thus, in Exp. 1, of 9·7 g. of oleyl alcohol administered, there has been excreted about 1·6 g., and in Exp. 2, of 4·53 g., about 0·3 g. It may be calculated from these figures that the amount of oleyl alcohol absorbed by the rat per day is 0·045 g. in Exp. 1, and 0·047 g. in Exp. 2.

Phytol. The phytol used was in part prepared from nettle leaves by the method of Willstätter and Stoll [1913]. The remainder was kindly supplied by Prof. I. M. Heilbron. The experiments were carried out as described for oleyl alcohol.

Exp. 3	No. of rats	Time days	Weight of livers g.	Weight of unsap. matter g.	Weight of cholesterol g.	Unsap. matter of faeces g.	Phytol administered cc.
Control diet	6	15	45·4	0·2308	0·1322	1·5586	—
Control diet + phytol	6	15	43·2	0·2400	0·1577	1·6338	4·80
Exp. 4							
Control diet	4	28	35·4	0·1316	0·1004	1·762	—
Control diet + phytol	7	28	61·0	0·3972	0·2762	1·828	7·47

From these results it appears that the phytol administered has been completely absorbed, and taking the figure of the first experiment, it is seen that the rat can absorb 0·046 g. of phytol per day.

Cetyl alcohol.

Exp. 5	No. of rats	Time days	Weight of livers g.	Weight of unsap. matter g.	Weight of cholesterol g.	Unsap. matter of faeces g.	Cetyl alcohol administered g.
Control diet	8	21	50·9	0·2206	0·1697	0·5703	—
Control diet + cetyl alcohol	8	21	46·1	0·1890	0·1290	1·1455	5·60

In Exps. 6 and 7, the animals received the basal diet plus 1 % of cetyl alcohol in an attempt to increase the amount of the unsaponifiable fraction of the liver.

Exps. 6 *and* 7	No. of rats	Time days	Weight of livers g.	Weight of unsap. matter g.	Weight of cholesterol g.	Unsap. matter of faeces g.
Control diet	6	16	45·4	0·1868	—	0·9546
	6	16	45·3	0·1561	0·1036	0·8419
Control diet + cetyl alcohol	6	16	42·7	0·2104	—	2·5166
	6	16	41·1	0·1928	0·1044	2·1488

The results of Exp. 5 show that 8 rats have absorbed about 5 g. of cetyl alcohol in 21 days, or an absorption of 0·030 g. per day. If we take 10 g. per day as the figure for the food intake of each rat, the total amount of cetyl alcohol ingested in Exps. 6 and 7 by 12 rats in 16 days is 19·2 g., whereas about 1·5 g. has been excreted. This would give an average absorption of about 0·09 g. per rat per day as against 0·03 g. in Exp. 5.

Cholesterol. A known weight of cholesterol was suspended in olive oil at 37° and three or four drops of the suspension were given to each animal daily. At the end of the experiment, the unused cholesterol was determined by the digitonin method, and the weight of cholesterol so determined subtracted from the weight taken.

Exp. 8	No. of rats	Time days	Weight of livers g.	Weight of unsap. matter g.	Weight of cholesterol g.	Unsap. matter of faeces g.	Weight of cholesterol given g.
Control diet	12	21	76·3	0·3308	0·2545	0·8556	—
Control diet + cholesterol	12	21	77·2	0·9657	0·9122	4·9013	8·30

These results show that about 4 g. or 50 % of the cholesterol administered has been absorbed, which corresponds to about 0·016 g. per rat per day, and that it has caused an increase in the amount of liver cholesterol to about three and a half times the control value.

In a repetition of Exp. 8, animals were given the basal diet with the addition of 1 % of cholesterol for a period of 16 days instead of administration of a daily supplement.

Exp. 9	No. of rats	Weight of livers g.	Weight of unsap. matter g.	Weight of cholesterol g.	Unsap. matter of faeces g.
Control diet	6	50·6	0·2341	0·1779	0·7494
Control diet + 1 % cholesterol	6	51·1	0·6755	0·5934	8·7934

It is seen that the liver cholesterol has again risen to about three times the control value. No records were kept of food intake, but allowing that each

animal consumed 10 g. of the diet per day, the amount of cholesterol ingested by the experimental group is about 9·6 g. with an excretion of 8 g., or an absorption of 1·6 g. by 6 animals in 16 days.

From these two experiments, it is clear that cholesterol is absorbed by the rat to the extent of about 0·017 g. per day, and that much of this cholesterol finds its way into the liver.

The results of these experiments show that all the alcohols administered have been absorbed but that the degree to which they are absorbed varies. It is naturally not easy to state with any accuracy from the results of such experiments as these the actual amount of any one substance which the rat can absorb, because a number of factors, such as whether the substance was given alone, as a suspension in olive oil, or mixed in the diet, and the ages and weights of the rats, need to be taken into consideration. There seems little to choose between the amounts of oleyl alcohol and phytol which have been absorbed and the results obtained in the experiments with these substances may be well compared, for each was given in drops. Apparently none of the phytol administered in either experiment has been excreted, so that the figure obtained for the absorption, namely 0·046 g. per day, must necessarily be a minimum. On the other hand, some of the oleyl alcohol has been excreted in both experiments with that substance, and hence the figure obtained for the absorption, namely 0·046 g. per rat per day, the mean of Exps. 1 and 2, is a maximum. This seems to show definitely that phytol is more readily absorbed than oleyl alcohol.

The results of the experiments with cetyl alcohol differ from each other. Where the alcohol was given as a suspension in olive oil, the rat absorbed 0·03 g. per day: on the other hand, in Exps. 6 and 7 of this series, in which the alcohol constituted 1 % of the diet, an average figure of 0·09 g. per day is obtained. There seems little doubt that the degree to which such substances as these are absorbed depends to a considerable extent on the method of administration and on what other fatty substances are present in the diet. Thus in their paper on Mutton Bird oil, which contains 38 % of unsaponifiable matter consisting chiefly of cetyl alcohol together with oleyl alcohol and a small amount of cholesterol, Carter and Malcolm [1927] found that when they administered the oil direct, 3 adult rats were able to absorb almost all of the 12·33 g. which they received in 8 days. This amount of oil contained 4·7 g. of unsaponifiable matter, *i.e.* the absorption of the unsaponifiable fraction was at the rate of 0·19 g. per day per rat, whereas when they administered cetyl alcohol as a suspension in olive oil only 0·056 g. per day was absorbed. In Exps. 6 and 7, in which cetyl alcohol constituted 1 % of the diet, the alcohol and the butter of the diet were melted up together before incorporation with the other constituents, and an absorption of 0·09 g. per rat per day resulted. The results obtained with cetyl alcohol confirm the observations of Thomas and Flaschenträger [1923] who found that a dog would absorb 4 to 6 g. of cetyl alcohol per day.

Cholesterol on the other hand seems to be absorbed to a much smaller

extent, namely at the rate of 0·016 g. per day. It seems safe to conclude, therefore, that of the alcohols used in these experiments, phytol is most readily absorbed; oleyl alcohol is next, and is followed by cetyl alcohol: cholesterol is absorbed to the least degree. It will be observed that the first two alcohols are liquid, whereas cetyl alcohol and cholesterol have M.P. 51° and 148·5° respectively.

Other considerations pointed to the fact that partial absorption of some of these substances might occur, for it is to be remembered that the animal depends for its existence on its ability to absorb vitamin A, which is an unsaponifiable substance, and probably a higher alcohol [Drummond, Channon and Coward, 1925]. Further, the animal can absorb vitamin D, which is again an unsaponifiable substance. The production of vitamin D by the action of ultra-violet light on ergosterol [Rosenheim and Webster, 1927] makes it appear possible that vitamin D is an unsaturated alcohol also.

Further, reference may be made to the lipochrome pigments carotene, $C_{40}H_{56}$, and xanthophyll, $C_{40}H_{56}O_2$. Of the earlier workers, Sauermann [1889] showed that plant carotinoids can be transferred to the feathers and egg-yolks of birds, and Willstätter and Escher [1912] demonstrated that certain of the animal lipochromes are identical with plant carotinoids. More recently Palmer [1922] and his colleagues have shown that the carotene of the butter-fat, adipose tissue and blood-serum of cattle is derived from the food, as is the xanthophyll of the tissues and egg-yolks of hens. These results have been confirmed by other workers and sufficient evidence seems available to make it certain that the lipochrome pigments present in animal tissues are derived solely from the diet, so that here again we have further examples of the absorption of a hydrocarbon of high molecular weight and its related oxygen compound, both of which are common constituents of plant material.

The unsaponifiable fraction of the liver.

The results previously recorded [Channon, 1926] showed that after absorption, squalene could be detected in the liver, and that the unsaponifiable fraction of that organ was increased to two or three times the control value. In Table I there are given the weights of unsaponifiable matter and of cholesterol calculated per 100 g. of liver from the foregoing results.

Table I. *Weights of unsaponifiable matter and cholesterol contained in* 100 g. *of liver.*

Experiment		Unsap. matter per 100 g. liver g.	Cholesterol per 100 g. liver g.		Unsap. matter per 100 g. liver g.	Cholesterol per 100 g. liver g.
1	Control	0·3883	0·3359	Control+ oleyl alcohol	0·5087	0·3666
2	,,	0·5250	0·2777	,,	0·5750	0·3737
3	,,	0·5083	0·2912	phytol	0·5555	0·3651
4	,,	0·3717	0·2836	,,	0·6511	0·4528
5	,,	0·4335	0·3336	cetyl alcohol	0·4101	0·2799
6	,,	0·4119	—	,,	0·4925	—
7	,,	0·3445	0·2287	,,	0·4691	0·2540
8	,,	0·4335	0·3336	cholesterol	1·2510	1·1810
9	,,	0·4625	0·3517	,,	1·3220	1·1610

Since all the control animals were receiving the same diet, an average value of the figures for all of them calculated on the basis of 100 g. of liver may be taken, and mean values taken also for the results of the experiments with each substance. These results are recorded in Table II together with the results obtained in the experiments with squalene [Channon, 1926].

Table II.

	Unsap. matter per 100 g. liver g.	Cholesterol per 100 g. liver g.
Controls	0·4310	0·3045
,, + oleyl alcohol	0·5419	0·3701
,, + phytol	0·6033	0·4089
,, + cetyl alcohol	0·4541	0·2669
,, + cholesterol	1·2865	1·1700
,, + squalene	1·1156	0·6437

The average figures for the nine control groups of animals are very close to those recorded previously [Channon, 1926] for an average of five groups which served as controls in the experiments with squalene, namely 0·4226 g. for the unsaponifiable fraction and 0·3179 g. for the cholesterol per 100 g. liver. There seems, therefore, some justification for considering these figures for the control groups as of significance, although the variations from them in three cases are greater than desirable. Compared with the average of 0·4310 g. for the unsaponifiable fraction of the control groups, the figures for the oleyl alcohol and phytol experiments, namely 0·5419 and 0·6033 g., seem definitely raised outside the variation obtained in the figures for the control animals. The results with cetyl alcohol, however, are inconclusive. The cholesterol experiments show that the unsaponifiable matter for 100 g. of liver has been raised to about four times the control value, and the increase is wholly accounted for by the rise in the amount of cholesterol in this fraction.

It seems justifiable, therefore, to conclude that the amount of the unsaponifiable fraction of the liver is increased when unsaponifiable substances are included in the diet. In the phytol experiment in which the amount of unsaponifiable fraction has increased, there appears to be a definite increase in the amount of cholesterol. The cholesterol figure in the oleyl alcohol experiment shows a similar, although smaller, increase. These results, however, may help to explain those obtained by administering squalene, when the figure for cholesterol rose to 0·6437 g., while the amount of the unsaponifiable fraction increased to 1·1156 g. They suggest possibly that when the amount of the unsaponifiable fraction of the liver is increased, the amount of cholesterol in that fraction shows a similar tendency to increase, rather than that squalene is a precursor in cholesterol synthesis in the animal body. Further experiments are being undertaken with a view to procuring additional evidence on this point, but it is to be observed that such a relationship would be the reverse of that which has been demonstrated in certain fish-liver oils [Channon, 1928].

The process of absorption.

We may now consider the possible methods by which these unsaponifiable substances are absorbed. Moore and Parker [1901] extended the early observations that bile would dissolve fatty acids by showing that the solubilities of such acids in bile salt solutions which contained lecithin were very much greater than in bile salt solution alone, and these results coupled with those of other workers have given rise to the modern view that fat is first hydrolysed by the pancreatic lipase, and that the resulting fatty acids are absorbed chiefly as a solution in bile. This suggested the desirability of determining the solubility of these unsaponifiable substances in solutions of bile salts. Commercial bile salts were purified by repeated solution in alcohol and crystallisation at 0° after the addition of ether. A 5 % solution was then shaken for 12 hours at 37° with excess of the different substances. The mixtures were then filtered through Whatman No. 3 paper at 37° to give clear golden brown solutions, 25 cc. portions of which were exhaustively extracted with ether, and the ether extracts, after washing with water, were evaporated to dryness *in vacuo* at 100°, taken up in ether, filtered, and weighed after removing the solvent. The mean values are given in Table III.

Table III. *Solubilities in* 5 % *bile salt solution at* 37°.

Phytol	Oleyl alcohol	Cetyl alcohol	Cholesterol	Squalene	Paraffin
%	%	%	%	%	%
>2·00	0·490	0·176	0·029	0·024	0·00

Not too great a reliance may be placed on these figures, for considerable difficulties arise in the determination of solubilities in bile salt solutions. Firstly, the solubilities and consequently the amounts weighed were very low and, secondly, the emulsions which arise when the ether solutions are washed with water render the values obtained somewhat uncertain. Results of the same order were obtained when the solubilities were determined in a solution containing 5 % of bile salt and 0·25 % of lecithin prepared by Levene's method [1927], which had N, 1·92 %; P, 4·06 %, the presence of the lecithin making no significant difference to the solubilities. It is of interest to observe from the above table that the degrees to which these substances are absorbed, as reported earlier in the paper, run parallel with their solubilities in bile salt solutions, save that squalene appears to be absorbed to a somewhat greater extent than cholesterol, for it was found in Exp. 1 of a previous paper [Channon, 1926] to be absorbed to the extent of 0·024 g. per day as against 0·017 g. per day for cholesterol. These results, therefore, would fit in well with the theory of the absorption of fatty acids—that solution in bile is the means whereby such substances are absorbed from the intestine. In this connection, the experiments of Mellanby [1927, 1] are of interest. Mellanby inserted cannulae into the gall bladder, common bile duct and pancreatic duct of a cat and introduced various fat emulsions into the duodenum. Fat absorption was

determined by inspection of the lacteals in the mesentery, and it was found that emulsified fat to which bile had been added was rapidly absorbed and caused a secretion of pancreatic juice and an increased flow of bile. On the other hand, neither emulsified fat nor fat previously digested by lipase was absorbed in the absence of bile, and the injections caused no secretion of pancreatic juice and no increase in the bile secretion. According to these results, therefore, emulsified fat in bile is absorbed by the cat without previous hydrolysis by lipase. Continuing these experiments Mellanby [1927, 2] reported that in a similar experiment in which an emulsion of paraffin and bile was injected into the duodenum of a cat there was neither absorption of the hydrocarbon nor secretion of pancreatic juice nor an increase in the amount of bile secreted. The author concludes from this result that the hydrocarbon had prevented the absorption of the bile salts by the intestinal mucosa. In view of this behaviour of liquid paraffin, Prof. Mellanby very kindly undertook to carry out a similar experiment with squalene. He injected squalene emulsified with diluted bile (2 cc. of squalene, 2 cc. bile, 5 cc. Ringer's solution, 1 cc. 1·5 % $NaHCO_3$) into the duodenum, and found by inspection of the lymphatics that it was absorbed to a small extent in the duodenum and upper part of the jejunum. Contrary to the behaviour of liquid paraffin, there was no interference with the absorption of bile as evidenced by the large secretion of pancreatic juice and bile following the injection. It seems clear from Mellanby's experiments both with emulsified fat and with squalene that particulate absorption of fatty substances may occur in the small intestine. Determinations of the solubility of olive oil in 5 % bile salt solution gave a figure of the order of that given by squalene. Although it is almost certain that the solubilities of these substances in bile itself will be considerably greater [Moore and Rockwood, 1897], Mellanby's results can only be interpreted as meaning that particulate absorption may occur. His failure to observe absorption of emulsified fat in the absence of bile suggests either that a sufficiently fine emulsion of the fat is produced only when bile is present, or, alternatively, that the bile salts may have a direct effect on the permeability of the mucosa of the small intestine. The latter view seems to us the more probable. The contrast in the behaviour of liquid paraffin and squalene when injected into the duodenum suggests that liquid paraffin is not absorbed on account of its lack of reactive groups. On the other hand, although the possession of such groups by a substance is a factor concerned in its ability to be absorbed, it is clear that there must be other factors, for otherwise these unsaponifiable substances should be absorbed to a much greater degree.

SUMMARY.

1. The ability of the rat to absorb phytol, cholesterol, oleyl and cetyl alcohols from the alimentary tract has been studied.

2. The degree to which these substances are absorbed is in the order phytol, oleyl alcohol, cetyl alcohol, cholesterol, the most easily absorbed being placed first.

3. The solubilities of these substances in bile salt solutions fall in the same order as the degrees to which they are absorbed by the rat.

4. The presence of unsaponifiable substances in the diet increases the unsaponifiable fraction of the liver.

5. Where such increase occurs, the cholesterol in that fraction tends to increase also.

6. The possibility of particulate absorption occurring in the small intestine is briefly discussed.

The authors wish to thank Prof. J. Mellanby, of St Thomas's Hospital, for his kindness in carrying out an experiment with squalene, and Prof. I. M. Heilbron of Liverpool University for his generosity in supplying them with phytol.

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LI. THE NATURE OF THE ACTION OF PANCREATIC EXTRACT ON THE INHIBITION OF LACTIC ACID FORMATION IN MUSCLE.

By DOUGLAS ROY McCULLAGH.
From the Biochemical Laboratory, Cambridge.

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The fact that the formation of lactic acid in muscle is inhibited by an extract from the pancreas was first observed by Winfield and Hopkins [1915]. Foster and Woodrow [1924] pointed out that the factor responsible for the inhibition was neither trypsin, insulin, nor Cohnheim's [1904] glycolytic factor; and they also suggested that it was not the antiglyoxalase of Dakin and Dudley [1913]. Foster [1925], after further work, which was however criticised by Dudley [1926], definitely concluded that the pancreatic factor was not antiglyoxalase. Since that time research in this laboratory by other workers [Reay, 1927; Downes, 1927] has made it seem desirable to add confirmation to portions of Foster's work.

Meyerhof [1926, 1] has recently demonstrated that under proper conditions the muscle enzymes responsible for the formation of lactic acid can be removed from the muscle in aqueous or saline solutions. This discovery opened new paths for the study of the pancreatic factor of Winfield and Hopkins. During the course of the research recorded in this paper Ronzoni [1927] published a preliminary report concerning the efficacy of the pancreatic factor in its action on the soluble enzyme system. Her results are in agreement with the preliminary results included in this paper, except that she states that there is no change in the inorganic phosphate when the formation of lactic acid by the enzymes from muscle is inhibited by the pancreatic factor. Her protocol, however, does not entirely bear out this statement.

Methods.

Preparation of the soluble lactic acid-forming enzymes from rabbit muscle is carried out as follows. The animal is anaesthetised by an intraperitoneal injection of amytal (75 mg. per kg. body weight). The hind legs are cooled in an ice-bath before killing the animal by bleeding. The muscles of the hind legs are then removed rapidly in a chamber at 0°, and minced in an ice-cold mincing machine. They are then extracted for half an hour with three times their weight of distilled water previously cooled to 0°. The extract is filtered through muslin and the filtrate used without further treatment. Fresh muscle extract was prepared immediately before use.

The following pancreas preparations are used.

(*a*) Desiccated pancreas as described by Foster and Woodrow [1924].

(*b*) One gram of (*a*) extracted for one hour at room temperature with 13·2 cc. of distilled water. This is made up to a total volume of 50 cc. with 95 % alcohol. The preparation is then completed according to Foster's directions, the final volume being 25 cc.

The following buffers are used.

(*a*) Phosphate buffer:

$NaHCO_3$	...	...	0·12 *M.*
K_2HPO_4	...	...	0·04 *M.*
NaCl	...	...	0·02 *M.*

(*b*) Fluoride buffer: the same as the above with the addition of NaF ... 0·10 *M.*

Methods of estimation. 25 cc. of muscle extract are placed in a flask together with 10 cc. of 2 % Lintner's soluble starch, 10 cc. buffer (phosphate unless otherwise stated), and 5 cc. pancreatic extract (or 5 cc. distilled water for the control flasks). After mixing, a sample of 15 cc. is removed at once and delivered into 20 cc. of 6 % hydrochloric acid, and a sample of 1 cc. delivered into 10 cc. of 10 % hydrochloric acid. The mixture is then incubated for 4 hours in a water-bath at 30°, and again sampled in the same fashion.

Proteins are removed from the 15 cc. sample by the addition of 25 cc. of 5 % mercuric chloride, allowing to stand over night before filtering. Saturated potassium hydroxide solution is added to the filtrate until a faint yellow permanent precipitate is obtained (usually 2·0 to 2·5 cc.). The mercury is removed with hydrogen sulphide and the latter by a current of moist air. Phosphates are estimated by the Briggs [1922] modification of the Bell-Doisy method, results being expressed as mg. of phosphorus in 15 cc. of the original mixture.

Sugars are removed from 30 cc. of the hydrogen sulphide-free filtrate by the addition of 10 cc. of 10 % calcium hydroxide suspension and 5 cc. of 10 % copper sulphate. Lactic acid is determined in the filtrate by oxidising with potassium permanganate, by the method of Meyerhof [1920]. Results are expressed as mg. of lactic acid in 15 cc. of the incubation mixture. In calculating the lactic acid and phosphate content no correction has been applied for the change in volume introduced during neutralisation with potassium hydroxide. This introduces a small error (2 cc. added to 60) which is partially compensated for by evaporation during the removal of hydrogen sulphide. This error, of course, is constant and occurs in every determination.

The 1 cc. sample is used for the determination of total carbohydrate as it was found that the starch present does not go through the filter quantitatively during the protein precipitation of the larger sample. The carbohydrates are hydrolysed by boiling on a water-bath for $2\frac{1}{2}$ hours, filtered and neutralised and the reducing power is estimated by the method of Hagedorn and Jensen [1923]. Results are expressed as mg. of glucose in 15 cc. of the incubation mixture.

Experimental.

The first portion of the investigation was conducted in order to confirm the fact that the inhibiting factor is not trypsin. Earlier workers, using muscle hash, caused 40 to 60 % inhibition in the production of lactic acid by the addition of pancreas. As can be seen from the tables below, when pancreas is added to the soluble enzyme system, formation of lactic acid is completely prevented. The difference is undoubtedly due to the elimination of factors such as permeability. The fact that the inhibition is complete and instantaneous would indicate a probability that the action is not due to tryptic proteolysis.

A 70 % alcoholic extract of the desiccated gland prepared carefully according to the instructions of Foster contained no trypsin but caused no inhibition. Dakin and Dudley [1913] stated that incubation for 4 hours at 37° destroyed trypsin. As pointed out by Foster [1925], this is not so; moreover, it was found that the inhibitor was partially destroyed during this treatment. However, preparation (*b*) above showed no tryptic activity during the course of an incubation at 30° for 4 hours and was quite active as an inhibitor as is shown by the Tables. Tryptic activity was tested for (*a*) by the biuret test on the protein-free filtrate after incubation, and (*b*) by the ability of the preparation to change dilute caseinogen solution to compounds not precipitated by acetic acid. The same method of preparation was used throughout.

That this factor does not act merely as an antiglyoxalase is shown by the following work which demonstrates that the inhibition in the formation of lactic acid is due to the failure of the system to form hexosephosphate. Mr J. B. S. Haldane has suggested the possibility that the same factor might prevent the formation of hexosephosphate and also act as an antiglyoxalase. If the mechanism is that of inactivating the co-enzymes, this dual action is quite possible.

During preliminary work it was noted that when the formation of lactic acid was prevented by the use of the pancreatic factor the total reducing power was not changed. Table I contains some typical results.

Table I.

	mg. lactic acid in 15 cc.			mg. total carbohydrates in 15 cc.		
	Before incubation	After incubation	Change	Before incubation	After incubation	Change
1. Control	10·6	22·1	+11·5	68·0	50·0	−18·0
Pancreas (*b*)	9·7	10·2	+ 0·5	70·0	70·7	+ 0·7
2. Control	6·8	19·1	+12·3	70·6	54·2	−16·4
Pancreas (*b*)	7·3	7·1	− 0·2	71·9	71·9	0·0
3. Control	6·1	11·9	+ 5·8	69·7	59·5	−10·2
Pancreas (*b*)	5·8	6·6	+ 0·8	67·9	67·3	− 0·6

The hexosephosphates differ from glucose in their reducing power and some of them are sufficiently resistant to mineral acid hydrolysis to withstand the treatment employed for the estimation of total carbohydrates. These results,

therefore, suggested that the hexosephosphates were not being formed in the presence of pancreatic extract. A quantitative study of the phosphate changes showed this to be true.

As pointed out by Meyerhof [1926, 2], during the formation of lactic acid by the soluble enzyme system in phosphate buffer, the free phosphate usually decreases. The experiments in Table II demonstrate this, and also show that when the formation of lactic acid is prevented by the pancreatic factor there is a slight increase in free phosphate.

Table II.

	mg. lactic acid in 15 cc.			mg. P as free phosphate in 15 cc.		
	Before incubation	After incubation	Change	Before incubation	After incubation	Change
1. Control	10·2	25·9	+15·7	5·81	5·38	−0·43
Pancreas (*b*)	9·7	9·9	+ 0·2	5·81	6·67	+0·86
2. Control	8·2	18·2	+10·0	5·78	3·83	−1·95
Pancreas (*b*)	8·4	8·4	0·0	5·85	7·14	+1·29
3. Control	8·5	16·4	+ 7·9	5·95	5·03	−0·92
Pancreas (*b*)	10·4	9·7	− 0·7	5·81	7·01	+1·30

These results indicate that in the control flasks hexosephosphates are formed more rapidly than they undergo lactic fermentation, whereas in the presence of pancreas hexosephosphates present in the muscle extract are broken down. Eggleton and Eggleton [1927] have shown that the Briggs modification of the Bell-Doisy methods for the estimation of phosphates determines also the phosphate present as phosphagen. Any possibility that the increase in the free phosphate, shown in these experiments, is caused by phosphagen hydrolysis, is eliminated since the method of estimation includes the phosphagen phosphate both before and after incubation.

Embden and Hayman [1924] showed that the addition of sodium fluoride to hashed muscle caused a marked decrease in free phosphate which was apparently transformed into hexosephosphate. Davenport and Cotonio [1927] demonstrated that the same thing occurred when sodium fluoride was added to the soluble enzyme system of Meyerhof. The results in Table III confirm those of Davenport and Cotonio and in addition show that in the presence of pancreas there is no disappearance of free phosphate.

Table III.

	mg. lactic acid in 15 cc.			mg. P as free phosphate in 15 cc.		
	Before incubation	After incubation	Change	Before incubation	After incubation	Change
1. Control	8·6	22·0	+13·4	5·9	3·6	−2·3
Fluoride	7·2	9·3	+ 2·1	5·9	1·3	−4·6
Fl. + pan. (*b*)	8·3	7·5	− 0·8	5·9	6·2	+0·3
2. Control	8·3	22·1	+11·8	5·9	3·5	−2·4
Fluoride	7·7	9·2	+ 1·5	5·6	1·2	−4·4
Fl. + pan. (*b*)	9·2	6·8	− 2·4	5·9	6·4	+0·5

SUMMARY.

(1) The result of former workers in regard to the presence in the pancreas of a substance which inhibits the formation of lactic acid by muscle enzymes has been confirmed.

(2) When the pancreatic factor is added to an extract of muscle, containing lactic acid-forming enzymes, it (*a*) prevents the formation of lactic acid; (*b*) prevents the disappearance of carbohydrate; (*c*) causes an increase in free phosphate content; (*d*) prevents the esterification of phosphate even in presence of fluoride. This demonstrates that the action of the pancreatic factor in inhibiting the formation of lactic acid is that of preventing the formation of hexosephosphates and not in preventing their fermentation.

Research concerning the physical, chemical and physiological properties of the pancreatic factor is in progress. Present results seem to indicate that this substance is present in traces in many other tissues from various species of mammals.

I am greatly indebted to Sir F. G. Hopkins for suggesting this problem and for his constant encouragement and to Miss E. Howland for assistance during this work.

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LII. THE SYNTHESIS OF 3:4-DIHYDROXY-PHENYLALANINE.

By CHARLES ROBERT HARINGTON.

From the Department of Pathological Chemistry, University College Hospital Medical School, London.

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In a note recently published in this *Journal* by the author together with W. McCartney [1927] on a modification of the Erlenmeyer amino-acid synthesis, a description was given of the synthesis of 3:4-dihydroxyphenylalanine from vanillin. My attention has since been called, by other workers, to certain difficulties which they have encountered in the preparation of the ethyl benzoylamino-3-methoxy-4-hydroxycinnamate from the azlactone resulting from the condensation of vanillin with hippuric acid. Although, in our hands, the method originally described gave satisfactory results, the preparation was admittedly tedious. The reaction was therefore re-investigated, and a much more rapid method of working up the ester has been devised. In view of the fact that the series of reactions under consideration appears to constitute the simplest available synthesis of 3:4-dihydroxyphenylalanine, it is thought worth while to record the following improved method of preparation of the ester.

10 g. of the azlactone, obtained by the condensation of vanillin with hippuric acid, were dissolved in 100 cc. of alcohol to which were added 10 cc. of concentrated sulphuric acid; the solution was boiled under a reflux condenser for 20 minutes. About 75 % of the alcohol was distilled off under diminished pressure, the bath temperature being kept below 40°; with this precaution, no discoloration should occur at this stage. The residual yellowish syrup was poured into a dish and rubbed up with cold water to which was added sufficient sodium bicarbonate to neutralise the sulphuric acid present and to leave the solution slightly alkaline. The oily ester was separated with the aid of ethyl acetate, the aqueous solution was again extracted with this solvent, and the combined ethyl acetate extracts were dried and evaporated. The residue was dissolved in warm alcohol and the ester precipitated by the cautious addition of water; it separated first as an oil which crystallised on rubbing; this crystallisation is almost immediate if a trace of the substance be available for seeding. After standing in the ice-chest for some hours, the precipitate was filtered off and recrystallised from dilute (60 %–65 %) alcohol. There was thus obtained 55 %–60 % of the theoretical yield of the pure ester, M.P. 129°.

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LIII. METABOLISM IN SCURVY.

I. THE LACTIC ACID EXCRETION OF SCORBUTIC GUINEA-PIGS.

By HENRY LEIGH SHIPP and
SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

Report to the Medical Research Council.

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As a result of a great number of investigations Bickel [1924, 1, 2; 1925, 1, 2] advanced the suggestion that "avitaminosis" in animals was associated with an "inner hunger." They arrived at this conclusion from the following observations. Animals (mostly dogs) subsisting on a diet free from vitamins gradually lost weight in spite of a liberal consumption of food, manifesting at the same time a decreased oxygen consumption. On the other hand, the C/N ratio of the urine increased. This was attributed to the presence of "dysoxydabler Kohlenstoff" originating from the partially oxidised carbohydrates which normally would have been metabolised to carbon dioxide. In consequence the problem arose of establishing the chemical identity of this "dysoxydabler Kohlenstoff" and reasoning from Neuberg's [1924] scheme of alcoholic fermentation it was considered that lactic acid formed by internal dismutation from methylglyoxal might be one of the probable constituents. With this hypothesis in view Rosenwald [1926] working in Bickel's laboratory investigated the lactic acid content of the urine of dogs on diets free from vitamins.

Rosenwald employed three animals, the first two received a diet of rice, wheat protein, lard and a salt mixture, whilst the third animal was given orange juice instead of lard. In the first two experiments there was about a fourfold to sixfold increase in the percentage and in the total output of the lactic acid in the urine after the animals had been receiving the deficient diet for 2–3 weeks. This increased excretion of lactic acid was not observed in the case of the third animal after 7 days, although the level of the lactic acid in this case was higher. The administration of insulin during the period of high lactic acid excretion brought about an *immediate* decrease in the C/N ratio of the urine, followed a day or two later by decrease in the lactic acid output. The author concludes that the disturbance in the C/N ratio of the urine and the abnormal excretion of lactic acid are due to a vitamin deficiency and that since

the animal receiving the orange juice showed no such deviation (*after 7 days*) it cannot be a vitamin A deficiency. She further expressed the opinion that it is most probably due to a vitamin B deficiency. The immediate diminution of the C/N ratio followed by decreased excretion of lactic acid after the administration of insulin is due, according to her, to the fact that lactic acid forms only one of the constituents of the "dysoxydabler Kohlenstoff."

It is evident that the above experiments are open to criticism in many respects. Concerning the diets, it would be more correct to state that the animals were suffering from malnutrition of a multiple character rather than from "avitaminosis," as their diets were imperfect, apart from the lack of vitamins. Whatever may have been the cause of the increased excretion of lactic acid in Rosenwald's experiments there was no justification for the conclusions drawn from her results.

Similar criticism may be levelled at most of the work done by Prof. Bickel and his school in this domain. To consider manifestations observed in animals existing on diets free from all the vitamins, even though perfect in other respects, as being due to a simple deficiency designated vaguely as "avitaminosis," is obviously wrong and consequently their experiments, apart from other imperfections, fail to lend support to the elegant hypotheses they advance.

In the course of an investigation on the metabolism in scurvy we had the opportunity of studying the lactic acid in the urines of normal and scorbutic guinea-pigs as well as of guinea-pigs suffering from general malnutrition. We were unable to observe any significant change in the percentage of lactic acid in the urine of the animals under the various dietetic conditions and in view of Rosenwald's observations on dogs we think it of interest to put our experiments on record.

Experimental.

The guinea-pigs were kept in modified Hopkins metabolism cages, which permitted the quantitative collection of the urine and faeces. In the scurvy experiments the animals received a mixture of oats and bran *ad lib.* and 40 cc. of autoclaved milk *per diem* and when necessary decitrated lemon juice as an antiscorbutic. A quantitative record was kept of the oats and bran consumed by the animals. The lactic acid was estimated daily in the urine by the Clausen method [1922]. After the preparatory ether extraction, the urine was brought up to 50 % sulphuric acid concentration, distilled at 145° in a metal-bath and the aldehyde estimated in the usual way. Although the quantities of lactic acid yielded in the urines were in most cases just on the border line of the limits of accuracy of the method, we have satisfied ourselves that any increase in the lactic acid concentration would have been registered. Thus the addition of lactic acid in the form of pure zinc lactate in quantities varying from 0·05–0·1 mg. to quantities of urine used in the estimation (2 cc.) produced a corresponding increase with an error of 8–10 % in the results. Hopkins's

thiophen reaction was obtained from ethereal extracts of 2 cc. of urine. The characteristic colour was, however, vitiated by the production of a brown coloration in the heating. An attempt to isolate lactic acid as zinc lactate from the urine of guinea-pigs failed when quantities of 100 cc. were employed. This was not pursued, in view of the negative character of the investigation. The urines gave no reaction for acetone bodies.

The lactic acid excretion of growing scorbutic guinea-pigs.

Six animals were utilised in this section of the investigation and the results are represented graphically in Fig. 1.

Guinea-pig No. 1 was on a scorbutic diet of oats and bran and autoclaved milk from the commencement of the experiment and, as is usual on this diet, it succumbed to scurvy after 25 days. It will be seen that the total daily outputs of urine and of lactic acid ran parallel. On the 20th day of the experiment, while the guinea-pig was declining in weight, there was a marked increase in the excretion of urine and of lactic acid which persisted until death. This phenomenon was observed on several other occasions and it will be shown that it is not due to scurvy. Polyuria in scorbutic animals has been previously observed by Lopez-Lomba and Randoin [1923].

Guinea-pig No. 2. The history and behaviour of this animal are similar to those of Guinea-pig No. 1 except that the polyuria in the premortal phase was not so marked. This animal received water *ad lib.* instead of milk.

Guinea-pig No. 3. This animal received, during the first 12 days, the basal diet supplemented by a daily dose of 5 cc. of decitrated lemon juice and after this time the scorbutic diet alone. Unfortunately the guinea-pig succumbed to an intestinal infection after having subsisted on the basal diet for 14 days only.

Guinea-pig No. 4 was treated as the preceding animal. In this one, however, death was due entirely to scurvy. The urine and lactic acid excretion during the scorbutic period was similar to that observed in Guinea-pigs Nos. 1 and 2 and showed no deviation from that in the prescorbutic period. It will be noticed that the animal did not grow during the time it received the anti-scorbutic. This was most probably due to the unaccustomed conditions of existence.

Guinea-pig No. 5 exhibited polyuria with the concomitant increase of lactic acid excretion to a marked degree in the premortal phase. It is to be noted that death was here due to an intercurrent disease, the animal suffering at the time of death from incipient scurvy.

Guinea-pig No. 6 was investigated during a prescorbutic period of 8 days, followed by a scorbutic period of 19 days, after which time the administration of decitrated lemon juice was resumed. The animal, however, never recuperated properly and had to be chloroformed 15 days later. In this case, as in the others, no evidence of an increased lactic acid excretion due to scurvy could be obtained.

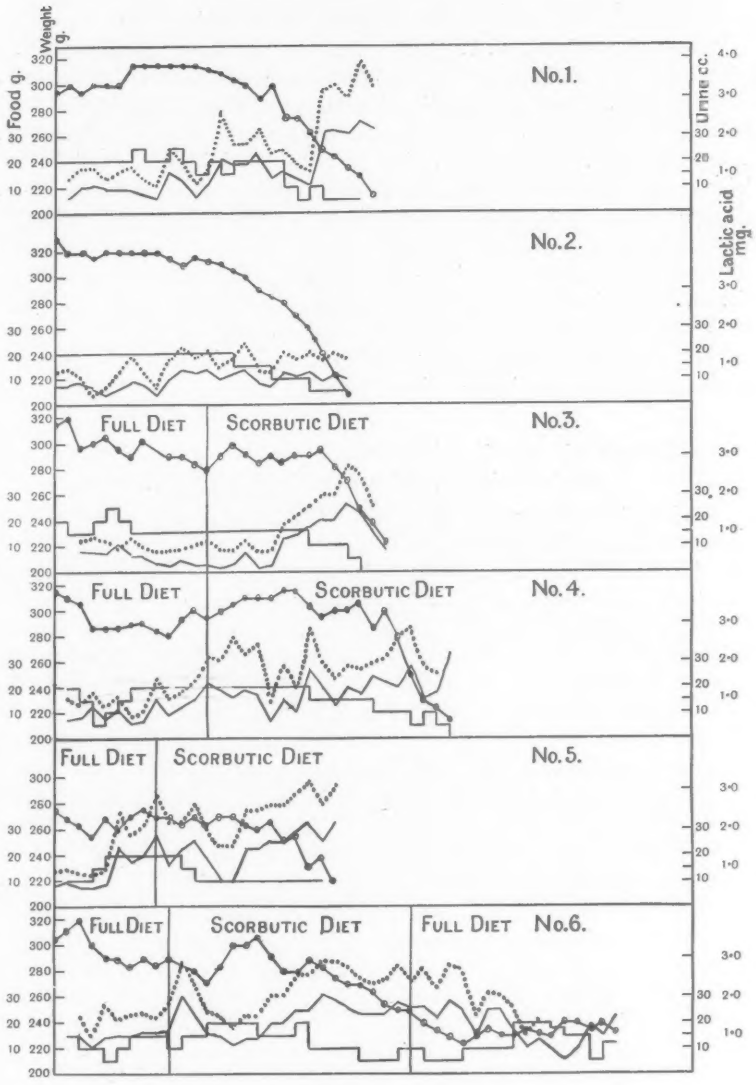

Fig. 1.

Weight o—o—o Volume ——
Lactic acid ••••• Food _┌¯¯┐_

In all the figures the points in the weight curves of the animals indicate the daily weights. The time scale of the other data in the diagrams is the same.

The lactic acid excretion of adult scorbutic guinea-pigs.

Three guinea-pigs (Nos. 7, 11, 12) were used in these experiments. The animals succumbed rather earlier than is usual with guinea-pigs of this weight, but in all cases scurvy was established at the post-mortem examination. The diet and management were the same as in the case of the younger guinea-pigs, except that 10 cc. instead of 5 cc. of decitrated lemon juice were administered daily during the prescorbutic period, and that this was replaced by 10 cc. of tap water during the scorbutic period in order to keep the quantity of liquid consumed as nearly as possible the same. With the exception of 4 days preceding death in the case of Guinea-pig No. 7, and 2 days in that of Guinea-pig No. 12, 40 cc. of autoclaved milk were consumed daily quantitatively. Fig. 2 gives the results obtained with these animals. The tendency to develop polyuria and a parallel increased excretion of lactic acid during the period of decline preceding death is also evident here.

The lactic acid excretion of growing guinea-pigs on a quantitatively restricted diet containing the antiscorbutic factor.

In view of the above results it remained to ascertain whether the tendency to increased excretion of urine during the decline in weight preceding death was characteristic of scurvy. Guinea-pigs were, therefore, placed on a diet of bran, water and decitrated lemon juice. The animals were weighed every day and the quantity of bran offered was such as to ensure a gradual decline in weight until the animal had lost rather less than a third of its original weight. 40 cc. of water and 5 cc. of decitrated lemon juice were administered daily with a pipette so that the volume of liquid consumed was kept constant. All the animals were observed during a preliminary period on a diet of oats, bran, 5 cc. decitrated lemon juice and 40 cc. of autoclaved milk.

The results are represented graphically in Fig. 3. It will be seen from the charts of Guinea-pigs Nos. 15 and 22 that almost simultaneously with the loss in body weight there appeared an increased excretion of urine. Guinea-pigs Nos. 16 and 24, after a period of 10 days on the restricted diet, were given again the mixed diet of oats, bran, cabbage and autoclaved milk. As these animals began to grow the volume of the daily urine also decreased and eventually reached the level observed in the preliminary period on the mixed diet. This is more marked with Guinea-pig No. 24. It is to be also noted that the lactic acid output of the animals Nos. 15 and 16, as in the case of the scorbutic guinea-pigs, ran parallel to that of the urine. In view of this observation the lactic acid excretion of the remaining animals of this set was not investigated further. No significant traces of protein were observed in the urine on addition of trichloroacetic acid during the polyuria in these or in the scorbutic guinea-pigs.

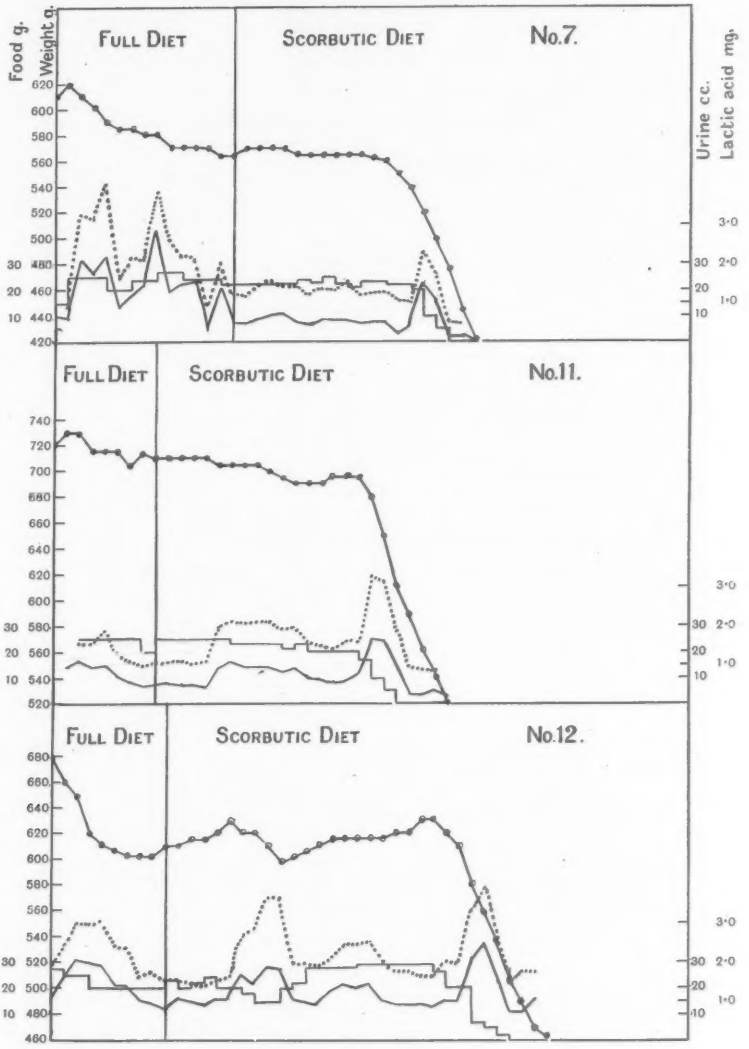

Fig. 2.

Weight o—o—o
Lactic acid .....
Volume ——
Food _|—|_

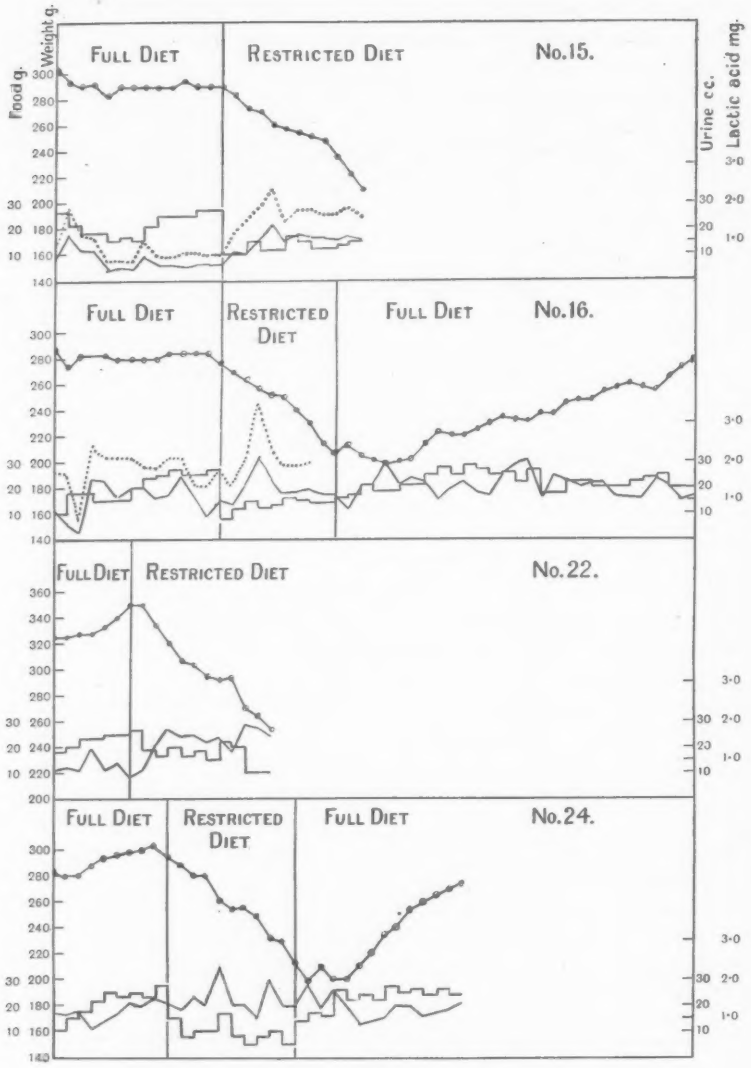

Fig. 3.

Weight o—o—o Volume ——
Lactic acid ····· Food _┌—┐_

SUMMARY.

The concentration of lactic acid (about 0·01 %), as established by Clausen's method in the urine of adult and young scorbutic guinea-pigs, is fairly constant. The decline in weight of the animals in the premortal phase is usually associated with polyuria. There is no increase, however, in the concentration of lactic acid in this condition nor is the polyuria associated with albuminuria. A similar increase in the volume of the urine takes place in guinea-pigs declining in weight on a restricted diet containing a sufficiency of the anti-scorbutic factor. It is, therefore, not specific to scurvy in guinea-pigs.

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LIV. PHOSPHORUS COMPOUNDS OF MILK.
IV. PRESENCE OF ADENINE NUCLEOTIDE IN MILK.

By HERBERT DAVENPORT KAY (Beit Memorial Research Fellow)
and PHILIP GUY MARSHALL.

From the Medical Unit, The London Hospital.

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It has been shown by one of us [Kay, 1925] that there are present in protein-free filtrates from goat's, cow's or human milk, acid-soluble phosphoric esters which are readily hydrolysable by dilute acids and by extracts of certain tissues, and are readily diffusible through collodion membranes.

We have now separated one of these compounds which appears to be composed of adenine and a pentose in combination with phosphoric acid. It is present in goat's milk, but only in very small quantities. So far, we have not been able to demonstrate its presence in cow's or in human milk.

Jackson [1923, 1924] brought forward evidence to show that adenine nucleotide was present in blood, but he was not able to obtain analytical figures indicating purity, and believed that his substance was contaminated with an approximately equal quantity of a pyrimidine nucleotide. Hoffman [1925] has isolated crystalline adenine nucleotide from pig's blood. We have ourselves obtained a substance from goat's and from pig's blood which gives reactions for adenine, pentose and phosphoric acid combined together. In milk, however, the similar compound is present in much smaller quantities than in pig's or in human blood (accepting Jackson's figure of 15–25 mg. nucleotide per 100 cc. for the latter). The highest figure yet found for goat's milk is 3·3 mg. nucleotide, corresponding with about 0·3 mg. phosphorus per 100 cc. milk. The average organic acid-soluble phosphorus of goat's milk is some 13 mg. per 100 cc. The nucleotide that we have found accounts therefore for only a small fraction of the ester phosphorus. It is in fact so small in quantity that it may be only an adventitious milk constituent, derived from cellular breakdown in the mammary gland.

Method of separation[1].

Goat's milk in 300 cc. lots was diluted to 500 cc. with water and shaken thoroughly with excess of picric acid paste. After standing 15 minutes the mixture was filtered, and the combined filtrates from 10 l. of milk evaporated *in vacuo* to about 2½ l. A crop of potassium picrate separated on cooling. The filtrate was neutralised to Congo red with 10 % NaOH, and filtered. 450 cc.

[1] Based on Jackson's method, after several unsuccessful attempts to devise a method giving a better yield.

of 20 % uranyl nitrate were added, and the whole was kept overnight. The precipitate was filtered off and washed twice with water by grinding in a mortar. It was suspended in water, and solution effected by adding $2N$ H_2SO_4. The filtered solution was treated with 10 g. $HgSO_4$ dissolved in $2N$ H_2SO_4, and 10 % NaOH added until a copious precipitate formed. Fractionation at this stage did not appear to be of value. The mercury precipitate was decomposed with ammonium sulphide, excess of the latter being boiled off. A few cc. of magnesia mixture were added to precipitate free phosphate and flocculate the colloidal sulphur which was usually present. The filtrate was evaporated on the water-bath to 50 cc., and a solution of 3 g. $AgNO_3$ added. The nucleotide came down in the silver precipitate, which was decomposed with H_2S and the filtrate evaporated to about 5 cc. *in vacuo* at 35°. 10 volumes of alcohol were added, the precipitate was filtered off and dried. It gave P = 11·32 %, N = 14·2 % in one case. The substance was dissolved in water, reprecipitated in presence of dilute acetic acid as the lead salt, decomposed with H_2S, again precipitated with alcohol and dried. Two different specimens gave P = 11·3 %, 11·16 %, N = 16·42 %, 15·38 % (calculated for adenine nucleotide, P = 8·93 %, N = 20·1 %). At this stage the amount of substance present was of the order of 0·1 g., so that further purification was difficult. Fractional precipitation from aqueous solution with alcohol was of no avail. Specimens prepared by slightly different methods from goat's milk had at this stage similar analytical figures, but showed too high a phosphorus and too low a nitrogen content for pure adenine nucleotide. After four attempts (employing the above method or modifications of it), in which 20 gallons of goat's milk were used in all, we did not succeed in obtaining a product which gave analyses agreeing with adenine nucleotide. The small yield of material precluded purification by crystallisation of the brucine salt.

The dry white powder thus obtained is readily soluble in water and gives the following reactions. (*a*) It is acid to methyl red. (*b*) It contains no free phosphate. (*c*) On boiling with strong HCl it gives a typical pentose reaction with orcinol, phloroglucinol or with Bial's reagent. The pigments produced with these reagents are soluble in amyl alcohol, the extracts showing absorption bands on the green or the red side of the sodium *D* line exactly corresponding with those given by an authentic specimen of arabinose, similarly treated. (*d*) The aqueous solution gives no precipitate with ammoniacal silver nitrate, but after heating for 2½ hours with $N/2$ sulphuric acid the reaction mixture gives, with excess of ammoniacal silver nitrate, a precipitate, from which, after decomposing with HCl and removing the silver chloride, a crystalline picrate may be obtained. After two recrystallisations from water, it has M.P. 292° and N = 31·2 % (calculated for adenine picrate, N = 30·8 %). A mixed melting point with an authentic specimen of adenine picrate is unchanged. The crystalline form of the two is identical. (*e*) In 2½ hours, $N/2$ sulphuric acid, which is stated to decompose adenine nucleotide completely in this time, removes about half of the organic phosphorus. (*f*) Kidney

phosphatase at 38° completely hydrolyses the ester phosphorus to inorganic phosphate in 4 hours. (*g*) There is no reduction on boiling with Fehling's solution.

It seems clear that adenine nucleotide is present, either mixed or in loose combination with an impurity containing more phosphorus and less or no nitrogen. The contaminating substance may be diphosphoglyceric acid (P = 23·4 %). It is probably not a pyrimidine nucleotide, as the percentage of phosphorus found is higher than that of any known nucleotide. For comparison, the nucleotide from 2 l. of pig's blood was prepared in a similar way. A substance was obtained containing P = 11·3 %, N = 16·0 %, and identical in all the above reactions with the milk compound.

Summary.

Adenine nucleotide, associated with some other phosphoric ester with similar precipitation reactions and solubilities, has been shown to be present in very small quantities in goat's milk. Its concentration is of the order of 3 mg. nucleotide per 100 cc. milk.

We wish to express our thanks to Dr F. R. Goss for a micro-analysis of adenine picrate.

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LV. ANTINEURITIC YEAST CONCENTRATES.

IV. THE FURTHER PURIFICATION OF YEAST VITAMIN B_1 (CURATIVE)[1].

By HENRY WULFF KINNERSLEY and RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

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This work is being communicated now for two reasons. Confirmation of a previous statement is needed that preparations of activity under 0·1 mg. per day pigeon dose can be obtained from yeast. Moreover the details of preparing these more active concentrates are required in explanation of an accompanying paper [Peters, Kinnersley, Orr-Ewing and Reader, 1928].

It must be stated at the outset that we are not yet in a position to say finally whether the curative substance in yeast is the same as the rice vitamin B_1 (protective), described by Jansen and Donath [1927]. Preparations of activity 0·027 mg. per day have been obtained in small amounts, but this does not yet reach the activity reported by the Dutch workers for the protection of their pigeons, namely 0·01 mg. per day. Owing to the heavy losses in the final stages, larger scale work is necessary for the solution of the problem.

Previous work. The principal points in this research up to the present may be briefly reviewed at this juncture. By the use of norite charcoal, following the removal of precipitates with lead acetate, barium hydroxide and mercuric sulphate, the curative factor in baker's yeast can be obtained in good yield at an activity of 0·5–1·0 mg. per day dose [Peters, 1924, Kinnersley and Peters, 1927] free from the thermostable vitamin B_2 for rat growth [Chick and Roscoe, 1927]. A highly active preparation of 0·08 mg. per day did not restore the loss of weight in pigeons fed upon polished rice [Kinnersley and Peters, 1925]. The successful preparation of these concentrates depends upon realising that the earlier stages are adsorption stages, an exact parallel to the case of the enzymes. (Some reflection upon the concentration of constituents present soon shows that this must be so.) The active principle can be obtained in solution in absolute alcohol[2] even at reactions not far removed from neutral, when it has been freed from some of the associated substances. This settles many of the discrepant statements in the literature as to solubility. It is to be noted that Jansen and Donath [1927] have found their rice vitamin B_1 soluble in

[1] The term "Yeast vitamin B_1 (curative)" is substituted for "torulin."

[2] Unless otherwise mentioned, alcohol means ethyl alcohol and "absolute" alcohol ordinary absolute alcohol throughout this paper.

absolute alcohol. The active principle is stable to the action of nitrous acid, but is destroyed by alkali, in confirmation of the work of others. The tedium of earlier methods led to a return to a careful study of pre-charcoal steps [Kinnersley and Peters, 1927], and to the introduction of two main improvements. These were the rapid removal of yeast gum by baryta in the presence of small amounts of lead and an accurate definition of the conditions for adsorption upon charcoal, p_H 7·0 being found to be the optimum under our conditions. In this manner it is now easy to concentrate quickly to an activity of 0·3–0·5 mg. per day. In fact this is the quickest way of obtaining material of this concentration which is known to us at present. The new technique eliminates the trouble which has formed the main difficulty of this work, namely the variability of different batches of yeast under the same treatment. Up to the stage 0·15–0·3 mg. the results can be considered to be reproducible. Beyond the stage of 0·1 mg., we have not defined the technique with the same success.

Present work. The results obtained in the present paper extend those which we have previously reported to a region of greater activity. We have also included certain experiments upon the stability of the active principle made at intervals during the last three years.

It is shown that though it was possible to obtain a concentration of part of the activity from material of 0·9 to 0·09 mg. per day by the further use of norite charcoal, the technique was uncertain. Further treatment with norite removed no more active substance from solution (*A* Exp. 1). It is therefore concluded that adsorption upon norite is due in the earlier stages to the presence of a co-adsorbent.

Recourse was therefore made to the previous technique of alcohol fractionation, by the use of which in an improved form it was found possible to work up all the aqueous HCl extracts of the charcoal to an activity which varied from 0·15–0·3 mg. per day. In order to eliminate loss, great care must be exercised in the removal of the last traces of metals and of sulphate. Further the solution must not be too acid for treatment with H_2S (*B* Exp. 2). The technique is reproducible down to this stage. It was stated in an earlier communication that no success had attended our attempts to apply the technique of the Dutch workers to our fractions. These workers employed a successive treatment with silver nitrate and baryta, working to a definite p_H, followed by fractionation with phosphotungstic acid and then by H_2PtCl_6 in alcohol. This was completed by a separation with the mixed solvents, alcohol and acetone, producing their crystalline vitamin B_1 of pigeon activity 0·01 mg. By translating their results for bondols to pigeons, upon the assumption that 5 bondol doses equal one pigeon dose, the average activity of their preparations at the various stages is as follows: after silver and baryta 0·4 mg., after phosphotungstic acid 0·150 mg. and after H_2PtCl_6 in alcohol 0·04 mg. per day. The material obtained by the alcohol fractionation of the *N*/10 HCl extracts is therefore about as concentrated as that which they obtained at the phosphotungstic acid stage. In the case of the yeast preparations, following the technique of

others [Seidell, 1922] we have often obtained fractionation by the use of silver nitrate, and in fact our most active preparation of 0·08 mg. per day was obtained with ammoniacal silver. It has also been known to us for some time that in many cases the greater part of the activity passed into the fraction precipitated between p_H 5·0 and 7·0 and was thereby concentrated (*C* Exp. 3). This result was not however always obtained, and we were led to abandon the technique for yeast, in the belief that we were dealing even in this case with an adsorption. A recent repetition of the use of silver (*C* Exp. 4) with material of activity 0·17 mg. has not led us to return to its use. Some of the activity is precipitated but the amount is variable.

We have been more successful with the use of phosphotungstic acid and of H_2PtCl_6. Making use of the charcoal purification step described in their latest description of the isolation of rice vitamin B_1 [Jansen and Donath, 1927] and modifying their technique of extraction, an increase of activity of 2–3 times to 0·08 mg. can be obtained with phosphotungstic acid, but at the expense of losses (*C* Exp. 5 and Table I). It would seem that precipitation by phosphotungstic acid is a genuine property of the active principle, because it has never been possible to regenerate any activity in the filtrates. There seems to be little doubt that there is a tendency to inactivate the preparations for the pigeons at this stage, especially where acetone is employed (Table I). The use of H_2PtCl_6 has been disappointing (Table II). Though in point of fact the most active preparations have actually been obtained by its use, the losses have been high and the yields variable. There has always been activity left behind in the filtrate. Several preparations of activity 0·04–0·05 mg. per day have been obtained in small amounts, and two of 0·027 mg.

Some properties of yeast vitamin B_1.

There have been included in this paper certain experiments which we have carried out during the course of the last two years upon the solubility and stability of the vitamin. It must be emphasised that the properties of the preparations change during the process of concentration. In a recent textbook [Pryde, 1928], there occurs the following statement, "Although both vitamins are soluble in water, B_2 is less soluble than B_1 in strong alcohol, acetone and benzene." This statement, in common with others of a similar type, in our opinion gives a wrong impression of the facts, and is really erroneous without some qualification as to the activity of the material to which it is applied. In the early stages of a fractionation, there is abundant evidence from the work of Seidell [1926] and others, that vitamin B_1 is thrown out upon alcohol precipitates together with the factors necessary for weight maintenance in the pigeon. At this stage, however, increasing acidification of the yeast extracts makes the factor more soluble in alcohol. At a later stage, after the removal of certain substances which are relatively insoluble in alcohol, vitamin B_1 is soluble in alcohol of a high concentration. We know of no evidence which shows that yeast vitamin B_1 at activity of 1·0 mg. is soluble in solvents other

than alcohol or water. The solubility in benzene at earlier stages, claimed by McCollum and Simmonds [1918], has been shown by Williams and Waterman [1926] to have been due to the presence of traces of alcohol and water. In our experience yeast vitamin B_1 of activity 1·0–0·03 mg. per day has never been found to be soluble in chloroform (acid or alkaline solution), in carbon tetrachloride, ether, acetone, or ethyl acetate. When fractionated from mixtures of benzene and alcohol or ether and alcohol in which the water was reduced to a minimum, these preparations are largely thrown out of solution. Much here also depends upon the acidity.

Stability to alkali. Alkali treatment has not given clear cut results. The general experience shows that vitamin B_1 is destroyed by alkali. Sherman and Burton [1926] have shown that the vitamin B (growth-promoting) in canned tomatoes at 100° suffers the following destruction in 1 hour: 90–100 % at p_H 10·9, 66 % at p_H 9·2, and 32 % at p_H 7·9. Our experiments show that one hour's heating (Table III) with $N/2$ NaOH completely destroys the activity of a preparation of 0·059 mg. activity. The vitamin is therefore unstable to alkali in the heat at this stage of activity. 10 minutes' heating with $N/10$ NaOH at an activity of 3·0 mg. does not interfere with the activity; one hour reduces it. Other factors appear to operate in rendering the factor more unstable to alkali. This was seen best in the case of the combined effect of alkali and alcohol. In one of the early experiments with an inactive preparation, making alkaline in the cold to thymolphthalein in alcoholic solution for a short time was sufficient to inactivate the whole of a preparation; with a purer concentrate of 1·0 mg. per day activity, no effect was produced by alkali at room temperature in alcoholic solution. In one case treatment with alkali and hydrogen peroxide caused complete inactivation. In acid solution hydrogen peroxide has no effect. It may be concluded that alkali is destructive to the vitamin, but that other factors may operate to accelerate the action. In practice it is advisable to carry out any alkaline stage at a low temperature in the absence of alcohol. The vitamin preparations can be best stored in 90–99 % alcoholic solution made more acid than p_H 2·0. One such preparation (of activity 1·0 mg.) has been kept under these conditions in 99 % alcoholic solution for a period of three years at room temperature, with no appreciable alteration in activity. The purest preparations, however, have been found to lose activity at room temperature in a few weeks under these conditions.

Oxidising agents. It is generally believed that vitamin B_1 is not susceptible to the action of atmospheric oxygen and is not affected by exposure to ultraviolet light or to ozone. This indicates a marked stability to oxidising agents.

It has been mentioned above that hydrogen peroxide in one experiment (Exp. 25) in alkaline solution and at room temperature produced rapid destruction. In acid solution, warming with hydrogen peroxide produced no decrease in activity. This was not surprising considering that treatment with permanganate in acid solution to a point at which no further reduction of the permanganate took place in one case produced no decrease in the activity of the

solution (Exp. 30). We can therefore confirm the great stability to oxidising agents. It was also found that heating with 5 % nitric acid produced no effect upon the activity (Exp. 31).

Reducing agents. This matter has not been thoroughly investigated. H_2S is a standard reagent in the course of the purification. It was found that the combined action of hot H_2S and alcohol was not destructive (Exp. 32), nor was treatment with tin and zinc (Exp. 33). In each case however only one experiment has been performed.

Comparison with rice vitamin B_1 (protective).

The rice preparation has been shown to be curative to pigeons in doses of 0·2 mg. by Eijkman [1927]. Our solubilities agree with the Dutch results, and the activity reached by the treatment with phosphotungstic acid and H_2PtCl_6 in alcohol is reasonably near their activity at a similar stage. In a previous paper, it was stated that the substances giving a reddish reaction by the Koessler and Hanke [1919] modification of the Pauly test did not fractionate with the curative activity. This is true but it is not entirely conclusive, as there is always considerable disappearance of activity produced by the use of these reagents. It is possible therefore that inactivated vitamin might be intensifying the colour given in the filtrates from the fractions. All the purer fractions contain small amounts of substances which give the Pauly reaction. An attempt was made to estimate the amount of substance producing a reddish colour still present in the most active fractions. This showed that if yeast vitamin B_1 is pure at 0·01 mg. per day (the rice figure), then the Pauly reaction which it gives is about one-fifth of the intensity of that given by an equal weight of histidine. More than this cannot be stated with certainty. The most active preparations when treated with mercuric sulphate give a slight precipitate. The Dutch preparations are stated to give a massive one.

Chemical nature.

Owing to the obvious impurity of the active preparations, not much attempt has been made to analyse them. Some of the fractions have been found to contain from 13–20 % nitrogen (Table IV).

The fractions show qualitative tests for sulphur, by fusion with sodium and treatment with sodium nitroprusside. Benedict's reaction is negative. The Molisch reaction is positive; judging approximately, a reaction was produced in the purest preparation equal to not more than one-tenth of its weight of cane sugar. The Pauly tests have already been discussed. The vitamin is much more stable to acid than is reported for co-zymase [Myrbäck and Euler, 1924], but it is interesting that co-zymase also is stable to treatment with permanganate [Euler, Myrbäck and Nillson, 1928]. When examined in ultraviolet light behind glass which excludes the visible rays, the less pure concentrates show a fine blue fluorescence. The purer concentrates[1], however, show

[1] We are indebted to Mr B. T. Squires for performing this test for us.

much less fluorescence than the filtrates from which they are separated, so that it is improbable that the active principle fluoresces under these conditions.

The possible existence of two forms of yeast vitamin B_1.

A number of conflicting results which we have obtained in the course of our work would be cleared up by the hypothesis that yeast extracts contain two forms of curative substance. The first example of diverse behaviour was a fractionation with neutral lead acetate following the charcoal stage. In material prepared commercially by the earlier methods, we always commenced with lead acetate. One of the functions of this step as we realise now was to remove the last traces of sulphate. It was always noticed that a certain proportion of activity, about 12 %, remained with the lead acetate precipitate, even upon a reprecipitation (Table V). The active substance so precipitated did not fractionate in quite the same manner as the remainder of the material. As the work has proceeded we have become aware of other cases, which could receive a similar explanation. The case of silver nitrate and baryta has already been discussed. Further, there is evidence to show that the active material extracted by 50 % acid alcohol from the charcoal is different in some way from that extracted by $N/10$ HCl. It is suggested that we are dealing with two forms of vitamin B_1, either differently combined or perhaps oxidised or reduced. We give the facts because possibly other workers in this field have had a similar experience.

Experimental.

Fractionation.

In practically all cases the material used for the further fractionation has been that obtained by extraction of the charcoal with $N/10$ HCl. The pigeon tests have been made by the method described elsewhere [Kinnersley, Peters and Reader, 1928]. The solid weights in all cases refer to organic solids.

Norite charcoal.

By the use of small amounts of charcoal upon the $N/10$ acid extracts after evaporating to a small volume, it was possible in some cases to adsorb part of the active substance at p_H 7·0, and to concentrate thereby 5–10 times. At one time it was hoped that this method would prove of value in the concentration, but later it was abandoned as the yields were poor and the steps impossible to define properly. The following experiment is illustrative of many carried out.

Exp. 1. $N/10$ HCl charcoal extract was concentrated to 50 cc. and found to contain about 5000 doses of activity 0·9 mg. NaOH was then added cautiously until the p_H was approximately 7·0. There was slight darkening of the fluid. 5 g. of norite charcoal were added and the whole was filtered. The charcoal was washed with a little water and extracted with $N/10$ HCl. The

extract was treated with alcohol for preservation. Solids, 0·129 g.; tests, pigeon doses, 1450; activity, 0·09 mg.

In this case about 30 % of the active material was separated by a very simple charcoal step giving a concentration of some 10 times. Further treatment of the filtrates from the second charcoal concentrates yielded hardly any more adsorption. It has been found to be an almost universal rule that the use of purified norite charcoal at a p_H acid to 4·0 clears the solutions of substances other than the vitamin. This holds down to an activity of 0·1 mg. per day. We thought that the rule was unbroken, but have recently used norite to clear a decomposed platinum precipitate (following Jansen and Donath). In this case much of the activity was picked out upon the charcoal, from which it could be recovered by warming with 50 % acid alcohol. The relations to charcoal of the highly active fractions (0·07 mg.) cannot therefore be said to have been defined with certainty.

The use of alcohol.

It is not possible to perform an alcohol fractionation upon the $N/10$ HCl extracts as they stand, because such trials are met with severe losses of activity. These have been found to be due to the combined effect of small traces of metals and of sulphuric acid. The losses can be avoided by treating with barium chloride in slight excess during concentration. The clearing of a charcoal extract from metals and the further fractionation with alcohol are carried out as follows. The details of an actual experiment are given together with the figures for the tests during the concentration. This is now a standard technique, and can be relied upon to give reproducible results.

Exp. 2. $N/10$ HCl extract of charcoal. The total volume of the extracting solution amounted to 800 cc. $BaCl_2$ (10 %) was added until no further precipitate appeared and the whole was concentrated to about 200 cc. More $BaCl_2$ was then added which brought down a further precipitate of sulphate. NaOH was then added cautiously until a p_H of 3·5 to 4·0 was reached. (This was roughly judged by the use of drops upon a plate with the indicator bromophenol blue.) H_2S was then passed for 2 hours. The large precipitate was removed by filtration. The filtrate was concentrated over the naked flame in a beaker to 30–40 cc. During the concentration, the H_2S is driven off and also much of the unpleasant mercaptan-like substance formed. To the small concentrate of some 30 cc., 80 cc. of 97 % alcohol were added. The whole was then subjected to the alcohol fractionation shown on p. 426.

The technique described can be relied upon to give material of activity about 0·15–0·3 mg. without loss of many doses. In this case it is further shown that charcoal at an acidity acid to Congo red does not extract the yeast vitamin B_1 (curative).

N.B. The alcohol precipitates are washed when they are flocculent. When gummy they are dissolved with minimal amounts of water and reprecipitated with alcohol.

Further fractionation of material of activity 0·1–0·3 mg. per day. Silver fractionation.

Work upon material of less activity than the above during intervals of the last 2 years has often given results in which the activity has fractionated as described by the Dutch authors. The results may be summarised by quoting the following experiment, illustrative of many.

Alcohol fractionation. Some 4000–5000 doses of activity (1·0 mg.).

N/10 extract concentrated over flame and finally *in vacuo* to about 20 cc., 140 cc. 97 % alcohol added.

Centrifugate. Concentrated to 8 cc. and 110 cc. alcohol added — 80/85 % alcohol precipitate

Centrifugate. Concentrated to 5 cc. and 115 cc. alcohol added. The precipitates were reworked with alcohol — 90 % alcohol precipitate

Centrifugate. Test dose. **4000.** Concentrated to 3 or 4 cc. + 115 cc. absolute alcohol — 94 % alcohol precipitate

Centrifugate. Concentrated to 1 cc. + 120 cc. absolute alcohol — 96 % alcohol precipitate

Centrifugate. Test dose **4800** — 99 % alcohol precipitate
Taken to dryness—NaOH added to p_H 5·0
To the volume of 6 cc., 170 cc. alcohol added — (Small precipitate removed)

Centrifugate. Concentrated to dryness. 2 cc. water added to dissolve and 120 cc. alcohol — 98 % alcohol precipitate

Centrifugate. 120 cc. (1·485 g. solids) — 99 % alcohol precipitate
Concentrated to a light gum, 0·5 cc. H_2O added and 150 cc absolute alcohol

Centrifugate. 150 cc. — 99 % alcohol precipitate, etc.
Removed the alcohol and treated the residue with a little water and enough HCl to make acid to Congo red. A small insoluble precipitate was removed by the centrifuge. The filtrate was treated with 5 g. charcoal and washed well. NaOH was then added to the filtrate from the charcoal to about p_H 6·0. The whole was concentrated to a light oil, and taken up in 150 cc. absolute alcohol

Final centrifugate 150 cc. (1·02) — 99 % alcohol precipitate, etc.

Tests $\begin{Bmatrix}2250\\4800\end{Bmatrix}$ **3500** average.

Activity 0·29 mg.

Exp. 3. 1926. Yeast concentrate of about 2·0 mg. activity, which had been treated previously with silver nitrite. About 480 doses taken and treated with silver nitrate solution until a drop gave a brown stain with baryta.

Precipitate
Test under 50 doses

Filtrate (acid to Congo red) treated with dilute ammonia to p_H 7·0

Precipitate p_H 7·0 extracted with HCl and alcohol
Pigeon test $\begin{Bmatrix}198\\99\end{Bmatrix}$ doses
Activity 0·3 mg.

Filtrate treated with HCl to remove silver
Pigeon test $\begin{Bmatrix}150\\150\end{Bmatrix}$ doses
Activity 2·0 mg.

About one-third of the activity was here concentrated in the fraction at p_H 7·0 with an increase of some 6 times.

Though we had often obtained the above result, there were many experiments in which it could not be obtained, and in which no precipitation took place. These were thought to be due to the presence of salts, which were accordingly removed by alcohol in some experiments, without however getting over the difficulty. This suggested that precipitation by silver was due to the presence of some associated substance. Since the publication of the Dutch work we have returned to the matter, and have subjected material of activity 0·17 mg. to a silver fractionation. Exp. 4 gives the details of the work.

Exp. 4. N/10 HCl extracts of the charcoal. 0·240 g. was taken, containing about 1400 doses in solution in absolute alcohol. The alcohol was removed and the residue taken up in 20 cc. water, 2 drops of 50 % sulphuric acid being added to make faintly acid to Congo red. Hot silver sulphate solution was then added until a drop removed gave a brown stain with baryta, then baryta until a reaction of p_H 4·0 was reached. The mixture was then centrifuged.

Centrifugate. Treated with baryta to p_H 7·4 and centrifuged — Precipitate (discarded)

Centrifugate treated with one drop of H_2SO_4. Evaporated to half its volume at p_H 5·0 and then brought to p_H 7·0 with baryta — Precipitate p_H 7·4. Extracted with two drops HCl and 5 cc. of cold water. Then with acid warm water. The combined extracts were treated with $BaCl_2$ to free from H_2SO_4 and brought to p_H 4·5 with NaOH

Centrifugate freed from silver with HCl and from barium with H_2SO_4. Alcohol added to a concentration of 50 %. Pigeon test 630 doses. Activity 0·4 mg. — Second precipitate extracted as first. Pigeon test 160 doses. Activity 0·2 mg.

Centrifugate. Evaporated *in vacuo* and taken up in alcohol. Pigeon test 350 doses. Activity 0·16 mg. — Precipitates discarded

It will be noticed that the doses recovered are about 1150 out of 1400, which is reasonable considering the variation of the bird test. It is clear that the main bulk of the vitamin remains behind and is not precipitated by silver-baryta under these conditions. The point to be noted is that the activity is no greater before than after the precipitation for the N/10 HCl extract. There is some evidence to show that silver-baryta fractionation is more selective in the case of the material extracted from the charcoal by 50 % acid alcohol.

Use of phosphotungstic acid.

As was briefly mentioned in a previous paper, a repetition of the first description given by Jansen and Donath [1926] for the use of phosphotungstic acid upon material of activity 0·4 mg. gave a recovery of no activity. In their further description [1927] it was found that a charcoal adsorption had been inserted before the phosphotungstic acid stage. By using the charcoal step, it was found that much of the activity could be recovered after precipitation with phosphotungstic acid, provided that the preparations were first treated with charcoal, and that extraction was performed by the

classical method of grinding the precipitates with solid baryta. Using this technique, however, the losses with the small amounts employed by us in these tests have still amounted to between 25 and 66 %. No activity remains in the filtrates, so that we feel satisfied that even at this stage of activity the whole of the active principle is precipitated by the phosphotungstic acid. The activity reached by the use of phosphotungstic acid appears to be about 0·07–0·08 mg. per day (about twice the activity obtained for rice by the Dutch workers). It is noticeable about these preparations that after treatment with phosphotungstic acid, the activity for *S. corallinus* (see accompanying communication, p. 434) always remains, whereas that for the pigeon is apt to be reduced. Some of the more salient features of the protocols are recorded below.

It may be mentioned that similar results have been obtained with a Merck preparation of phosphotungstic acid, as well as with a Kahlbaum preparation repurified by Winsterstein's method.

One experiment (Exp. 5) is given in detail to show the method which we have found most effective. The remaining experiments are recorded in Table I.

Exp. 5. Preparation containing originally approximately 4500 doses. Activity 0·33 mg. Removed alcohol, added 85 cc. H_2O and HCl to bring to p_H 1·0. Treated with 1 g. norite charcoal, allowed to stand for 1½ hours and filtered on a Büchner funnel, the precipitate being washed with $N/10$ HCl. To the filtrate 20 cc. 10 % phosphotungstic acid in 5 % H_2SO_4 were added. The whole was allowed to stand 48 hours, then centrifuged and the precipitate washed once with water. The precipitate was ground up 4 times with solid baryta, and the filtrate collected by centrifuging and filtering. Excess barium was removed by sulphuric acid, and the whole acidified with HCl to p_H 2·0. A trace of $BaCl_2$ was left in excess. The whole was concentrated *in vacuo* and extracted with 50 cc. absolute alcohol. The average doses were 2250, and the organic solids 0·300 g. making an activity of 0·13 mg.

Where reprecipitation of the phosphotungstates from acetone solution was carried out, the usual technique for such treatment was followed. By reprecipitation, we have obtained a slight increase in activity, but with considerably more loss.

Table I. *Phosphotungstic acid.*

	Doses			Activity			Methods	
Exp.	Taken	Recovered	% recovery	Initial mg.	Final mg.	Increase in activity	Charcoal	Acetone reprecipitation
6	2500	1064	42	0·6	0·2	×3	+	+
7	3000	1500	50	0·6	0·25	×2·4	Nil	+
8	700	350	50	0·34	?	?	+	+
9	1700	380	22	0·3	0·16	×2	+	+
10	1700	580	30	0·3	0·087	×3·5	+	+
5	3000	2250	75	0·33	0·13	×2·5	+	Nil
11	240	168	74	0·19	0·074	×2·5	+	Nil

NOTE. *Exps.* 9 and 10 were made with a brewer's yeast preparation; yield here especially bad: the remainder with baker's yeast. The yield is higher in Exps. 5 and 11 in which no acetone was allowed to touch the preparation, but the final activity is not quite so good as in Exps. 6 and 10. In the case of Exp. 10, all filtrates and precipitates obtained were freed from phosphotungstic acid and tested. They were completely inactive.

Chloroplatinic acid.

According to the Dutch workers, using H_2PtCl_6 at an activity for the pigeon of 0·150 mg., they can concentrate to an activity of 0·04 mg. (some 4 times), without much loss of the active material.

Table II summarises most of the results which we have obtained. The procedure has been in practically all cases identical with that employed by the above workers. H_2PtCl_6 has been added to the solutions in absolute alcohol. The precipitate so formed has been removed by the centrifuge, taken up in water and treated with H_2S. It has then been left to stand, and the precipitate of sulphide removed by filtration. It was found impossible to remove this completely in one stage. After heating upon the water-bath, fresh separations of the sulphide appeared which were removed. Finally the whole was taken down to dryness, in many cases finishing in the vacuum desiccator, and taken up in absolute alcohol. The concentration obtained will be seen to vary. By the double treatment with phosphotungstic acid followed by H_2PtCl_6, the activity was increased to 0·04 mg. In two cases 0·025 mg. was reached. We made one attempt to clear the platinum sulphide filtrate by the use of a small amount of norite (as described by the Dutch workers). To our surprise the charcoal (even at an acid p_H) extracted much of the activity, and by a re-extraction of the charcoal with 50 % acid alcohol highly active material of 0·026 mg. per day was obtained, in yield of about one-third of the final activity recovered by precipitation.

Table II. H_2PtCl_6 *in alcohol.*

	Doses				Activity			
		Recovered						
Exp.	Taken	Precipitate	Filtrate	% in precipitate	Initial mg.	Final mg.	Increase in activity	Remarks
12	260	75	60	29	0·15	0·2	Nil	No previous treatment with phosphotungstic acid
13	630	470	60	74	0·25	0·037	×7	Filtrate from which a precipitate had been removed with Ag and Ba (see Exp. 4)
14	1500	678	200	45	0·25	0·087	—	After phosphotungstic acid (see Exp. 7)
15	1064	240	210	22	0·20	0·10	×2	After phosphotungstic acid (see Exp. 6)
16	350	200	Nil	57	Unknown	0·27	?	After phosphotungstic acid treatment (see Exp. 8). Concentrations: Exps. 8 and 16 = 10 times
17	700	290	80	41	0·32	0·03?	Under 10 times	Only *one* experiment. No previous phosphotungstic acid treatment
18	960	275	—	28	0·12	0·06	×2	After phosphotungstic acid treatment (Exps. 9 and 10). Loss by using charcoal
19	2250	500	—	22	0·13	0·05	×2·6	After phosphotungstic acid treatment (Exp. 5). Loss by using charcoal

The charcoal used to clear 18 and 19 was extracted with warm 50 % acid alcohol; 500 doses were recovered of activity 0·026 mg. per day.

Oxidising agents.

Permanganate. Several experiments were carried out. In most of these some inactivation occurred, but in the one described below the condition was realised in which extensive permanganate oxidation took place without vitamin destruction.

Table III. *Action of alkali.*

(*a*) *Alkali alone.*

Exp.	No. doses taken	Activity mg.	Treatment	Doses recovered	Inactivation
20	10	0·059	Boiled 1 hour *N*/2 NaOH	Nil	Complete
21	11	3·0	Boiled 10 mins. *N*/10 NaOH in nitrogen	8	Slight
22	11	3·0	Boiled 10 mins. *N*/10 NaOH in air	7	Slight
23	11	3·0	Boiled 60 mins. *N*/10 NaOH with oxygen passing	2	Marked
24	11	1·0	Heating with ammonia (0·55 0)	Nil	Complete

(*b*) *Alkali and hydrogen peroxide.*

25	4	1·0	Treated with 1 drop of 25 % NaOH in 1 cc. H_2O and 4 drops of H_2O_2. Made acid with HCl after standing 15 mins. Heated to remove H_2O_2.	Nil	Complete

(*c*) *Alkali and alcohol at room temperature.*

26	—	—	Very impure concentrate made alkaline to thymolphthalein (10–15 mins.) in presence of alcohol and found to be completely inactivated.	Nil	Complete
27	4	1·0	Made alkaline in 60 % alcohol to thymolphthalein at room temperature for 15 mins.	Nil	Complete

(*d*) *Alkali and alcohol* 50 % *at* p_H 5·5–7·4.
Control 5 days. Activity 1·0 mg.

Exp. 28. *Standing at room temperature*

p_H	Stood	Days recovered
5·5	6 days	2·5
6·0	6 days	3 +
6·5	18 hours	4 –
6·5	8 days	1 –
6·5	8 days	Nil
7·2	11 days	3 –

Exp. 29. *Heating.*

p_H	Days recovered	
6·0	5 +	Evaporated 15 mins. in long tube
6·5	Nil	Evaporated 5 mins. in open tube
6·5	4	Boiled in long tube*
7·2	Nil	Heated 5 mins. on water-bath
7·4	2	Heated 1·5 hours on water-bath

* To avoid contact with air.

We are indebted to Miss Reader for carrying out the alkali treatment in Exps. 20–23.

Exp. 30. *Action of permanganate.* 300 pigeon doses of activity 1·0 mg. were treated in acid solution with 60 drops of strong potassium permanganate solution until no further reduction occurred. After this treatment the bird test gave 240 doses still present, an amount equal to the original within the limits of the test.

The action of nitric acid. Seidell [1921] makes the statement that the activity of some of his preparations diminished upon standing in the desiccator. This he attributed to the presence of nitric acid. The following experiment shows that we have not found the presence of dilute nitric acid dangerous to the activity. This would be anticipated from Tsukiye's [1922] results with a rice polishing concentrate.

Exp. 31. 0·2 cc. which contained about 5 day doses. Activity 1·5 mg. Samples of 0·2 cc. were concentrated to remove alcohol. Two drops of normal

HCl were added and 2 cc. of 5 % HNO_3. The whole was then heated on the water-bath for 10 minutes. The tests gave 8 and 10 days. If anything there was therefore increase in activity.

Reducing agents.

Exp. 32. H_2S *in alcoholic solution.* This experiment was done in order to find out whether addition of alcohol to an extract which still contained sulphuretted hydrogen was dangerous.

0·2 cc. containing 5 day doses in about 3 cc. of 75 % alcohol was treated with H_2S. The H_2S and alcohol were then boiled off and the whole concentrated to a small bulk. Two birds were tested and both gave 4 days, indicating that the loss was less than 30 %.

Exp. 33. *Reduction by zinc and tin in* HCl *solution.* After acting upon a preparation of 1·0 mg. with tin, followed by zinc in HCl solution, bringing to the neutral point and centrifuging off the large precipitate, the pigeon test showed the presence of the major part of the curative activity.

It may be concluded from the above that the material is fairly resistant to the action of reducing agents.

Pauly reaction [Koessler and Hanke, 1919]. This was performed as described by Koessler and Hanke, in small test-tubes. To 2·5 cc. of the sodium carbonate solution was added 1 cc. of nitrite reagent, the whole mixed and, one minute afterwards, this was poured into 0·5 cc. of the unknown solution and thoroughly mixed. The unknowns were compared against known amounts of histidine hydrochloride solution. 0·005 mg. (5 γ) gave a visible pink reaction, with 10γ the reaction was definite. The tests upon active preparations of yeast vitamin B_1 always showed much yellow. By adding small amounts of the preparations to known amounts of histidine hydrochloride solution, it was possible to show that the vitamin concentrates did not inhibit the production of the reddish coloration. They however masked it. Traces of a substance giving a reddish reaction were present in all the active samples. The amount present was roughly estimated to be equivalent to an amount of histidine hydrochloride equal to 2γ (0·002 mg.) per pigeon dose. If therefore yeast vitamin B_1 is the same as rice vitamin B_1 with a pigeon dose of 0·01 mg., then the reddish reaction given by vitamin B_1 must be about one-fifth of the intensity of that given by an equal weight of histidine.

Table IV. *Nitrogen.*

Source of material	Activity mg.	Total N %	N per dose mg.
Baker's yeast *N*/10 HCl extract of charcoal	0·09	20	0·018
" " " "	0·026	13·5	0·0035
Brewer's " " "	0·31	19·0	0·059
Baker's " 50 % alcohol extract of charcoal	0·10	12·7	0·013

Table V. *Charcoal concentrates prepared by earlier methods, showing constant precipitation of portion of activity by neutral lead acetate.*

Exp.	Doses taken	Doses recovered lead precipitate	Doses recovered lead filtrate	Notes
Q 1	2800	350	2000	The lead precipitate was extracted with HCl and alcohol, the alcohol removed, and reprecipitated
Q 3	3300	510	2750	No reprecipitation. Material had been treated with nitrite
S 1	—	450	3600	
S 5	—	300	2400	

In each case there is a definite precipitation by the neutral lead acetate, amounting to some 12 %. All precipitates were of course very thoroughly washed in each case.

The preliminary treatment consisted in removing alcohol from the 50 % acid alcohol extract of the charcoal, adding water and then treating with neutral lead acetate.

Note upon the behaviour of brewer's yeast.

We have recently had the opportunity of testing the behaviour of extracts of brewer's yeast to our latest methods of purification. The yields which we have obtained by our standard technique have been within the limits of those obtained with baker's yeast, but upon the low side. At present only the $N/10$ HCl extracts of the charcoal have been investigated.

Summary.

1. Preparations of yeast vitamin B_1 (curative) have been separated from the concentrates, which have reached in two cases an activity of 0·027 mg. per day (pigeon dose).

2. The technique for this separation can be considered to be reproducible down to an activity of 0·1 mg. per day.

3. Phosphotungstic acid and H_2PtCl_6 can be used for concentrating yeast vitamin B_1.

4. The solubility of vitamin B_1 in ethyl alcohol varies with the activity of the preparation and with the hydrogen ion concentration of the solution treated with the alcohol.

5. Vitamin B_1 so far as has been investigated is not soluble in chloroform, carbon tetrachloride, ether, acetone or ethyl acetate. This confirms the statements of others.

6. One hour's heating with 0·5 N NaOH destroys the curative powers of the vitamin completely at an activity of 0·06 mg. 10 minutes' heating with 0·1 N NaOH does not inactivate it appreciably. The vitamin may become more unstable to alkali in the presence of other substances.

7. The stability to oxidising agents is confirmed. Certain reducing agents do not destroy the activity.

8. It is suggested as a hypothesis that two forms of yeast vitamin B_1 occur in an aqueous extract of yeast.

9. Brewer's yeast can be used for obtaining the charcoal concentrates. The yields have not been quite as high as the best obtained from baker's yeast.

We are indebted to the Medical Research Council for a grant to one of us and for a grant for expenses. We are also grateful to W. Wakelin for help with the preparations and with the pigeons. We wish to record our thanks to Messrs Lewis, Clarke and Co. (Worcester) who have generously placed brewer's yeast at our disposal. We also acknowledge gratefully the help which has been obtained from the *S. corallinus* tests performed by Miss Reader and Miss Orr-Ewing, at various stages of the fractionation.

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LVI. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

III. THE EFFECT OF THE ADDITION OF GROWTH-PROMOTING SUBSTANCES TO THE SYNTHETIC MEDIUM ON THE GROWTH OF *STREPTOTHRIX CORALLINUS*.

By VERA READER.

From the Department of Physiology and Biochemistry, University College, London, and the Department of Biochemistry, Oxford.

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In a previous paper [1927] it was shown that certain organisms could be grown in a synthetic salt-sugar medium provided that the concentration of the inorganic ions was kept within moderately narrow limits: but it was soon observed that the addition of the smallest amounts of trypsin-digested beef broth to the synthetic medium had an enhancing effect on the three organisms under experiment (*Sarcina aurantiaca*, a white *Streptothrix*, and *Streptothrix corallinus*). In fact, the growth was so much more prolific than in a purely synthetic medium that it was decided to make a detailed study of the nature of the stimulating substances in broth, and to seek for other sources.

It was reported by Mueller [1922], and by Freedman and Funk [1922] that there are present in beef and in ox-heart infusions certain substances which show a strong growth-stimulating activity on haemolytic streptococci. Mueller showed that the activating material may be divided into two fractions, active only when mixed together, by means of fractional precipitation of the first $HgSO_4$ precipitate by $HgSO_4$, or precipitation by Ag_2SO_4 and baryta. Various workers have from time to time suggested that vitamin B or its associated substances might be responsible for the stimulation [cf. Thjotta and Avory, 1921; Kuroya and Hosoya, 1923].

Very many workers have studied the sources and properties of growth-stimulating substances for yeast, but in this paper bacterial factors only are considered; for a review of the literature on the bios question see Tanner [1925]. In order to avoid confusion in nomenclature the term *bacterial growth-promoting factor* has been used throughout, thus reserving the term *bios* for the factor augmenting the growth of yeast, as described by Wildiers [1901].

Doryland [1916], Mueller [1922] and others have discussed the possibility that unexpected relationships or dissimilarities in bacterial species may develop on the basis of food requirements, so it was decided to use one test organism only—*Streptothrix corallinus*—throughout this set of experiments. Later it is hoped to extend the studies to other species. The results recorded below, and by Orr-Ewing and Reader [1928] show that for this organism, at least, a growth-promoting factor is required. This is an organic water-soluble substance occurring in fresh beef, wheat embryo, and yeast, and closely associated though not identical with the antineuritic vitamin[1]. The author takes this opportunity of modifying a statement made at the British Association Meeting in 1926 to the effect that the bacterial growth-promoting factor was inactivated by alkali. This is often true for comparatively inactive concentrates. However the more active torulin concentrates (Kinnersley and Peters) can be shown to possess a stimulant power even after the inactivation of their antineuritic constituent (vitamin B_1) by alkali. Such antineuritic concentrates therefore show a bacterial growth-promoting activity, but the reverse is not true.

Experimental.

The preparation of the synthetic medium and the general conditions for growth are described in Part I [Reader, 1927]. After 48 hours' incubation at 31° growth was arrested by the addition of formalin and estimated by opacity, standard flasks of a definite extract (trypsin-digested ox-heart) in varying concentrations being used for comparison.

20 cc. inorganic medium + 0·5 % glucose + 0·1 cc. broth = vigorous.
20 cc. inorganic medium + 0·5 % glucose + 0·01 cc. broth = good.
20 cc. inorganic medium + 0·5 % glucose + 0·005 cc. broth = fair.
20 cc. inorganic medium + 0·5 % glucose + nil = slight.

The standard broth was prepared by the method of Douglas [1914]. A fresh bullock's heart, after removal of fat and vessels was finely minced. To every 500 g. of mince, 1 l. of distilled water was added and the liquid made faintly alkaline to litmus. After heating slowly for 5 minutes at 75° it was cooled to 37° and 1 % liquor trypsinae co. (Allen and Hanbury) added. It was then kept at 37° for 3 hours. In order to see that the digestion is complete, the fluid should be tested for peptone by the biuret reaction. It was then made slightly acid with glacial acetic acid, brought slowly to the boil and kept boiling for 15 minutes. After standing overnight in a cool place, it should be syphoned off, made *faintly* alkaline to litmus, placed in a steamer for ½ hour and then brought to the required p_H (usually 7·0) and sterilised for 20 minutes on three successive days. It is very important that at no stage in the preparation should the p_H be allowed to rise above 8·5. Reference will be made to this later.

[1] The relation of this factor to the antineuritic vitamin is fully discussed in an accompanying communication [Orr-Ewing and Reader, 1928].

Relation of growth to amount of added growth-promoting factor and effect of age of beef before preparing broth.

The following experiment was designed to show at what low limit of concentration of tryptic broth the enhanced effect on growth could be observed. Inorganic medium and 0·5 % glucose were used throughout and varying amounts of broth added. There is some indication that the active substance in beef-tissue is destroyed or inactivated on keeping, since broth prepared from ox-heart fresh from the butcher on the day of killing was more potent than that prepared from ox-heart which had been allowed to stand at room temperature (18°) for 2 days. However once the broth is made and sterilised it may be kept in the laboratory for 3 months (p_H 6·5–7·0) without an apparent loss of activity.

1 cc. of broth represents 0·5 g. fresh heart muscle. In Table I, *A* = broth from ox-heart, fresh; *B* = broth from ox-heart, after standing at room temperature for 2 days; *C* = as *A*, but kept in laboratory for 3 months at p_H 6·5.

Table I.

Concentration of broth in synthetic medium	Growth		
	A	*B*	*C*
1 : 20	vigorous	vigorous	vigorous
1 : 40	,,	good	,,
1 : 200	,,	slight	,,
1 : 400	,,	,,	,,
1 : 1000	very good	,,	very good
1 : 1500	good	,,	good
1 : 2000	fair	,,	fair
1 : 4000	slight	,,	slight

Slight growth, as will be seen from the list of standards, is equivalent to the growth obtained on a salt-sugar medium alone, *i.e.* there is no enhancement of the growth.

Physical properties of the growth-promoting factor.

(*a*) Water soluble; (*b*) insoluble in ether (see Table II); (*c*) dialysable (see Table II) through collodion sacs.

Table II.

Supplement to synthetic medium	Growth
(1) Control (salt-sugar)	slight
(2) Broth (1 in 200)	vigorous
(3) Ether extract of broth (equivalent 1 in 200)	slight
(4) Broth after extraction (equivalent 1 in 200)	vigorous
(5) Dialysed broth (1 in 200)	vigorous
(6) Dialysate (1 in 200)	slight

Chemical nature of the factor.

It was now decided to test the possibility of an inorganic salt or salts being responsible for the enhancing effect of broth, and also to test the inorganic portion of the bacteria themselves. (*a*) 20 cc. of broth *A* (Table I) and (*b*) 5 g. (approx.) of the organisms were ashed in a platinum dish using an Argand

burner. At three stages—(1) after heating to dryness; (2) after charring (approx. temp. = 170°); (3) after heating at a dull red heat to constant weight—an aliquot part (= 1 cc. original broth, or 0·25 g. bacteria) was taken out and tested for stimulating power.

In Table III these three parts are designated as Preparations I, II, and III.

Table III.

Medium	Growth
(1) Inorganic medium + glucose (control)	slight
(2) Control + 1 cc. broth in 20 cc.	vigorous
(3) Control + 1 cc. broth Prep. I.	vigorous
(4) Control + 1 cc. broth Prep. II.	good
(5) Control + 1 cc. broth Prep. III.	slight
(6) Control + 0·25 g. heated bacteria Prep. I.	vigorous
(7) Control + 0·25 g. heated bacteria Prep. II.	good
(8) Control + 0·25 g. heated bacteria Prep. III.	slight

The general conclusion from this table is that the substance is organic but appears only partially destroyed at the charring point (170°).

Precipitation of proteins, etc. An attempt was now made to remove from the broth all substances precipitated by mercuric chloride, lead acetate, or phosphotungstic acid, and to test the filtrate for growth-promoting power. Precipitations were carried out as follows.

(1) With $HgCl_2$: the excess Hg was precipitated with H_2S and the filtrate boiled until free of gas.

(2) With lead acetate: the excess lead was removed as sulphide and the filtrate boiled.

(3) With phosphotungstic acid in 5 % H_2SO_4 and treatment with $BaCl_2$.

Micro-Kjeldahl estimations before and after the precipitations showed that the N of 1 cc. of broth was lowered from 2·3 mg. to 0·3 mg. by all three methods.

Table IV.

Supplement to synthetic medium	Growth
(1) Broth (*A*) 1 cc. in 20 cc.	vigorous
(2) Filtrate after $HgCl_2$ (equivalent 1 in 200)	nil
(3) Filtrate after lead acetate (equivalent 1 in 200)	vigorous
(4) Filtrate after phosphotungstic acid (equivalent 1 in 200)	vigorous

Thus in two out of the three cases the active substance was still in the filtrate. Possibly one is not justified in assuming that a chemical separation could take place in any such precipitations, as the actual quantity of the factor present is probably so small that the conditions of adsorption on the flocculent precipitate may be the determining factor. However it seems unlikely that the inactivity of the filtrate after the $HgCl_2$ is due to the bactericidal action of traces of mercury in the solution as experiments have shown that concentrations of $HgCl_2$ less than 0·000025 % (25γ) do not cause inhibition.

The growth-promoter may have been precipitated by the $HgCl_2$, but tests of the precipitate failed to demonstrate this (see also the effect of $HgCl_2$ on torulin, Kinnersley and Peters [1925]). Possibly the factor was adsorbed on the mercuric sulphide precipitate, but so far it has never been recovered from this.

Other sources of this bacterial growth-promoting factor.

Aqueous extracts of the following animal and vegetable substances were prepared and tested to find what weight of raw material was necessary to give growth equal to 1 g. beef tissue (1 in 200 dilution being used as control).

	g.
Ox heart	1·0
Rabbit muscle	1·5
Serum	5·0
Yeast	0·2
Apple	No effect
Wheat germ	0·25
Crude thyroid gland	10·0
Purified thyroid	No effect
Impure glucose	10·0
Glucose	No effect

To ascertain whether a concentrated extract of yeast would have a greater growth-promoting effect than marmite, a sample of freshly prepared torulin concentrate [Kinnersley and Peters, 1925] was tested in dilutions of 1 pigeon day dose in 200 cc. to 1 in 20,000,000 cc. of inorganic-glucose medium. Even in concentrations of 1 in 2,000,000 a fair growth-promoting effect was obtained for *S. corallinus*. Since this preparation had an activity of 1/10 mg. per day dose the actual concentration of solid matter added was 5×10^{-7} mg. per 100 cc. To get the same degree of growth it is necessary to add 0·7 mg. weight of broth solids per 100 cc.

Effect of p_H *on the activity of the factor.*

In Table I it was shown that broth could be kept at p_H 6·5 for 3 months without loss of activity. Experiments were now arranged in which sufficient NaOH was added to two portions of broth to make them 1/10 *N*. One was kept at room temperature (18° approx.) for 3 months and the other was boiled for 1 hour. In both cases the activity was decreased to 10 % of its former value. A similar experiment with marmite revealed the same result. However with a much purer product the activity remained practically constant even when boiled with *N*/4 NaOH for 60 minutes (see Table V). The extract used in this experiment had an activity for the pigeon of 0·2 mg. *per diem*.

Table V.

	Volume of solution necessary to give growth equal to 0·5 cc. broth
1. Control (xxx)	0·050
2. Control boiled *N*/200 NaOH for 5 mins.	0·050
3. Control boiled *N*/40 NaOH for 5 mins.	0·045
4. Control boiled *N*/4 NaOH for 5 mins.	0·045
5. Control boiled *N*/200 NaOH for 60 mins.	0·045
6. Control boiled *N*/40 NaOH for 60 mins.	0·045
7. Control boiled *N*/4 NaOH for 60 mins.	0·045
8. Control autoclaved $p_H = 10$ for 30 mins. at two atmospheres	0·050
9. Control autoclaved $p_H = 7$ for 30 mins. at two atmospheres	0·050
10. Control autoclaved $p_H = 2$ for 30 mins. at two atmospheres	0·050

Thus it would appear that the bacterial growth-promoting property of the torulin concentrate is stable to alkali. The only other possibility is that some inhibitory substance was formed by the action of NaOH on the more crude broth and yeast, thereby masking the growth-promoting activity. That in this property of stability to alkali the bacterial growth-promoting factor for *S. corallinus* differs from the antineuritic vitamin will be considered again in an accompanying paper [Peters, Kinnersley, Orr-Ewing and Reader, 1928].

SUMMARY.

From these results it may be concluded that the substance in tryptic beef broth that exerts a stimulating action on the growth of *S. corallinus* is organic, water-soluble, and dialysable and is not precipitated by either neutral or basic lead acetate. It is also present in yeast preparations, rabbit muscle, serum, and wheat embryo. In the fractionation of the antineuritic vitamin from yeast this bacterial growth-promoting factor is associated with the vitamin up to the 1/10 mg. per pigeon day dose stage, *i.e.* the addition of 1×10^{-7} mg. of this preparation in 20 cc. of medium increases the growth 200 times. To what extent Funk and Dubin [1920] were justified in claiming to estimate quantitatively the presence of vitamin B by growth of micro-organisms other than yeasts will be considered in the next paper [Orr-Ewing and Reader, 1928].

In conclusion the author takes this opportunity of thanking Profs. J. C. Drummond and R. A. Peters for their advice and criticism and the Medical Research Council for a grant.

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LVII. *STREPTOTHRIX CORALLINUS* IN THE ESTIMATION OF VITAMIN B_1.

By JEAN ORR-EWING and VERA READER.

From the Department of Biochemistry, Oxford.

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This work was undertaken as an attempt to find a substitute for the laborious technique involved in the pigeon tests.

Williams [1919] first described a method of testing for the antineuritic vitamin which was based on the assumption that the yeast cell is capable of multiplication only in the presence of this factor. The test was, however, later discarded when Funk and Dubin [1920] demonstrated that the yeast stimulant (bios) was not identical, but merely associated, with the antineuritic factor.

Experimental.

When testing the more active torulin concentrates (0·1 mg. per day pigeon dose), a very considerable difference in growth was noted between flasks containing 1/20 and 1/40 dose, or again between those containing 1/400 and 1/800 dose in 20 cc. medium. It was then found that, given a fraction, say 1/1000 part, of a torulin concentrate, it was possible to predict the number of doses to be found in the whole. Hence we developed the following technique. It has been found capable, with certain limitations, of replacing the pigeon tests in following the fractionation of the vitamin.

Technique of the test.

Materials. 1. Synthetic medium prepared as described by Reader [1927].

2. *Streptothrix corallinus* [Reader, 1926]. A young culture should be used.

3. A torulin concentrate of which the pigeon dosage is known. A dilution is made up in absolute alcohol of such a strength that 0·1 cc. = 1/20 pigeon dose. This preparation is kept permanently as a standard for comparison with the samples under test.

4. Conical flasks of 50 cc. capacity. These must be scrupulously cleaned between each estimation, using hot chromic-sulphuric acid mixture and large quantities of distilled water. Both the flasks and pipettes needed should be reserved for use in the test only.

Setting up. The experimental flasks are set up as follows.

1. Medium 20 cc. minus the volume of the test fluid, which is carefully washed into the flask. (Dilution 1/20.)

2. Medium 19 cc. + 1 cc. carried over from Flask 1. (Dilution 1/400.)
3. Medium 19 cc. + 1 cc. carried over from Flask 2. (Dilution 1/8000.)

Standard flasks. Nine dilutions of the standard torulin are put up for comparison with the above. A dilution is first made by taking 0·75 cc. of the standard preparation, evaporating to dryness on the water-bath, and washing into 30 cc. of the medium. 0·1 cc. of the standard contains 1/20 pigeon dose. This amount is now contained in 4 cc. of the dilution *A*.

The nine flasks are now prepared as follows:

Flask	Medium		Standard dilution *A*	Actual dilution (of pigeon dose)
1	4 cc.	+	16 cc.	4/400
2	12 cc.	+	8 cc.	2/400
3	16 cc.	+	4 cc.	1/400
4	18 cc.	+	2 cc.	1/200
5	19 cc.	+	1 cc. from 1	1/2000
6	19 cc.	+	1 cc. from 2	1/4000
7	19 cc.	+	1 cc. from 3	1/8000
8	19 cc.	+	1 cc. from 4	1/16000
9	19 cc.	+	1 cc. from 5	1/40000

It will be seen that the dilutions in standard flasks nos. 3 and 7 correspond respectively with those in test flasks nos. 2 and 3, the remaining flasks containing multiples or submultiples of these quantities.

All flasks are steamed for half an hour on three successive days, and subsequently incubated for 24 hours at 31° to test for sterility. Each flask is then inoculated by loop from a suspension of *S. corallinus*. This is most conveniently taken from a young culture (2 to 6 days) grown on a medium corresponding to that in standard flask no. 9. The amount of growth is estimated at the end of 48 hours' incubation at 31°.

Reading the test. The amount of growth in test flask no. 2 is compared with that in the standard flasks nos. 1–5, and that in test flask no. 3 with standards 5–9. Readings in two dilutions are thus obtained over a range from 4 times to 1/5 of the strength of the standard concentration. Readings outside this range, obtainable on one flask only, should not be relied upon.

It has unfortunately not been possible to produce a uniform suspension of the organism. Nephelometric or other accurate methods of comparing growth cannot therefore be used, and only a very approximate estimate can be made. Experience has shown, however, that the average readings in the two flasks give a result of an accuracy comparable with that of the pigeon test.

The flasks are read both before and after shaking. Flask no. 1 is not used for the comparison of growth. It serves the purpose of detecting inhibitory agents, and certain stimulating substances other than the growth factor to which reference is made below.

Example. Test fluid = 0·65 cc. from a batch of 65 cc. (1/100 part).

Flask no. 1. Good.

Flask no. 2 = Standard flasks nos. 3–4.

Flask no. 3 = Standard flasks nos. 7–8.

Test fluid is therefore < 1 and $> 0{\cdot}5 = 0{\cdot}75$ of the standard (*i.e.* unit dose) strength. The whole batch of 65 cc. therefore contains $0{\cdot}75 \times 100 = 75$ doses.

Exceptional readings. Departures occur from this type of result; these are found to fall into two groups.

1. *Inhibition.* Certain reagents used in the extraction process, particularly mercury, silver and platinum salts, markedly inhibit the growth of the organism. Warning of this effect is always given by the poor growth in flask no. 1. Should the effect be still apparent in the higher dilutions, the estimation must be disregarded, or an approximation obtained from flask no. 3.

2. *Anomalous result.* A more serious source of error is the occurrence of an abnormally rapid falling off of growth as the dilution is increased.

Example. Test fluid = 1 cc. from a batch of 500 cc.

Flask no. 1. Very good.

Flask no. 2 = Standard 1 (standard $\times$ 4).

Flask no. 3 = Standard 8–9 (standard $\times \frac{1}{2}$ to $\frac{1}{5}$).

On the flask no. 2 reading therefore the batch would be calculated to contain 2000 doses, but, on the flask no. 3 reading, less than 200 doses. The pigeon test may, in these cases, give results approximating to the higher or lower or to an intermediate value of the *S. corallinus* readings. No information of value can therefore be obtained from results of this type. They occur, fortunately, only in the early stages of the yeast extraction, before charcoal adsorption. The value of the test in the later stages is therefore not impaired.

These anomalous results suggest that some other stimulating factor is present in the crude extracts. The nature of this substance is at present under investigation by one of us (Vera Reader).

The practical value of the test is discussed in a later paper [Peters, Kinnersley, Orr-Ewing, and Reader, 1928], where tables showing the parallel *Streptothrix* and pigeon tests will be found.

Summary.

A method of obtaining quantitative growth-promoting tests has been developed, which is applicable to the testing of antineuritic concentrates after the charcoal adsorption stage. A method is given of eliminating erroneous results due to the presence of inhibitory agents such as metals.

Certain anomalous results are obtained with material tested before the charcoal stage.

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LVIII. NOTE ON THE *MENINGOCOCCUS* AS A SOURCE OF GROWTH FACTOR FOR *STREPTOTHRIX CORALLINUS*.

By JEAN ORR-EWING and VERA READER.

From the Departments of Biochemistry and Pathology, Oxford.

(*Received March 1st, 1928.*)

The findings reported below have been demonstrated at the British Association meeting, 1926, and at a meeting of the Biochemical Society, June 1927. They seem to be of sufficient interest to be briefly summarised, as one of us (J. O.-E.) is unable at present to continue the work.

In the course of an endeavour to ascertain whether vitamin B_1 was essential to the growth of the haemophilic group of micro-organisms, it was discovered that a strain of *Meningococcus* type 1 (National collection of Type cultures, Strain Littledale, No. 668) would grow readily upon a meat extract medium which had been completely freed from the *S. corallinus* growth factor by repeated charcoal extractions. Since the *S. corallinus* has been found to react to a vitamin B_1 concentration of 2×10^{-6} pigeon dose [Reader, 1928], we are convinced that the vitamin B_1 factor, if present at all, was reduced below this value. The final test of the ability to grow in the absence of the vitamin lies in the production of growth in a carefully purified synthetic medium. We have been unable to find such a medium capable of supporting growth, and were unaware at the time of the work of Hosoya and Kuroya [1923].

On retesting the meat extract (vitamin B_1-free) medium after the growth of the *Meningococcus*, it was found to be capable of supporting good growth of *S. corallinus*.

Experimental.

Ordinary meat extract made from 500 g. of lean veal per litre of tap-water was extracted three times with 2 g. charcoal (norite) per 100 cc. To the filtered extract were added 0·4 % Na_2HPO_4 and sufficient 4 % NaOH solution to bring to p_H 7·2. The fluid was then tubed and autoclaved for ten minutes at one and a half atmospheres pressure. The *Meningococcus* was subcultured three times on this medium and five tubes were finally inoculated for use in the *Streptothrix* test [Reader and Orr-Ewing, 1928]. After six days' growth, the tubes were steamed and the contents added to synthetic medium as shown below.

Medium		*Streptothrix* growth	
Broth cc.	Synthetic medium cc.	Uninoculated medium	Inoculated medium
10	+10	Trace	Good = 1/100 pigeon dose
5	+15	,,	Moderate = 1/400 dose
1	+19	,,	Poor = 1/4000 dose
0	+20	,,	—

Similar results have been obtained with the centrifuged culture medium from which the bacteria were as far as possible removed.

It will be seen that the degree of *S. corallinus* growth falls off more quickly than is warranted by the dilution of the medium. The result is therefore of the anomalous type [see Reader and Orr-Ewing, 1928], and for this reason we do not claim to have demonstrated that the *Meningococcus* is able to synthesise vitamin B_1. The results are necessarily expressed in terms of pigeon doses, because this is our only measure of the degree of *S. corallinus* growth. An attempt has been made to test this point by extracting a large volume of the medium after the growth of the organism, but the concentrate was found to be too toxic for use in a pigeon test.

Discussion.

Numerous cases have been reported of the ability of the more hardy organisms such as *B. coli* and *Sarcina lutea* to produce growth stimulators for the delicate organisms such as *Meningococcus*, *Streptococcus haemolyticus*, *Pneumococcus* and *Gonococcus* [Ainley Walker, 1922; Hosoya and Kuroya, 1923]. This case is of some interest as an example of the reverse process. The bearing of this work upon the results of the Japanese workers is discussed in the following paper.

Our thanks are due to Prof. Dreyer for allowing us to carry out part of this work in the Department of Pathology.

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LIX. THE RELATION OF VITAMIN B_1 TO THE GROWTH-PROMOTING FACTOR[1] FOR A *STREPTOTHRIX*.

By RUDOLPH ALBERT PETERS, HENRY WULFF KINNERSLEY, JEAN ORR-EWING and VERA READER.

From the Department of Biochemistry, Oxford.

(*Received March 1st, 1928.*)

In this paper it is proposed to consider together the relevant facts from the two parallel researches of the preceding papers. A preliminary account of this work has already appeared elsewhere [see Peskett, 1926]. Though the work is clearly related to the bios problem [see the review by Tanner, 1925, and Eddy, Kerr and Williams, 1924], it is probable that a different substance is involved in these effects. The most extensive investigation of the relation between the water-soluble vitamin and bacterial growth factors is that by Hosoya and Kuroya [1923]. They concluded that bacteria could be divided into two main groups. In one group, containing the *Streptococcus haemolyticus* and *Pneumococcus*, an unknown water-soluble substance was absolutely necessary for growth in a synthetic medium. For the other, which included most bacteria tested (including the *Meningococcus*), addition of the growth factor was not absolutely necessary. The purest sample of antineuritic vitamin used by these workers was Tsukiye's vitamin B (from rice), which from their figures would appear to have had an activity in our sense of about 1·0 mg. per day. It was shown that the activity of the preparation in curing pigeons (vitamin B_1 effect) was readily lost by the preparations upon heating to 140° for 2 hours in neutral solution. On the other hand, the yeast growth stimulant in the preparation, bios, withstood a similar temperature in *N* NaOH solution. The *Streptococcus* factor occupied an intermediate position. It was destroyed by heating for 2 hours in *N*/40 NaOH solution. Among the other interesting points, it was found that a strain of *Meningococcus* could be grown upon Fraenkel's solution containing glucose and *d*-glutamic acid or *l*-histidine hydrochloride; this medium did not contain the growth factor for *Streptococcus*. Further, it was shown that a strain of *B. coli* which did not need this growth-promoting factor could synthesise it; an alcoholic extract of *B. coli* was capable of stimulating the growth of *Streptococcus*. *B. coli* grown upon a vitamin-free medium contained enough vitamin B_1 to cure pigeons, therefore the organism could synthesise vitamin B_1. This latter result was contrary to that of Braddon and Cooper [1914].

[1] The nomenclature recently recommended by the Accessory Food Factors Committee of the Medical Research Council (1928) is adopted throughout.

The results communicated here are concerned with an intensive study by parallel tests of two factors, the vitamin B_1 curative for pigeons, and a bacterial growth stimulant for *Streptothrix corallinus* [Reader, 1926].

Experimental.

The organism studied has been grown upon a synthetic medium with addition of vitamin concentrates, previously standardised upon the pigeon, and the methods of testing are fully described in the previous papers. It must be emphasised that the growth obtained upon this medium by the addition of the best vitamin B_1 concentrates (with an addition of as little as 4×10^{-5} mg. %) has been quite as vigorous as that upon undiluted broth medium.

Table I shows that, after a certain stage in the fractionation is reached, treatment with alkali removes the curative property but does not depress the growth-promoting effect for *S. corallinus*. At an earlier stage the factor for *S. corallinus* is less stable to alkali.

Table I. *Action of alkali.*

Exp.	Material	Activity* in mg.	Percentage decrease in activity: *Streptothrix* %	Pigeon %	Normality NaOH	Treatment and time heated at 100° mins.
1	Filtrate from Hg	50·0	100	—	0·1	60
2	Stock H	3·0	60	80	0·1	60 (oxygen passing)
3	„	3·0	Nil	Nil	0·1	10 („ „)
4	XXV	0·15–0·20	Nil	—	0·1	10 („ „)
5	„	0·15–0·20	Nil	50	0·1	10 („ „) (marmite added)
6	XXX–XXXIII	0·09	Nil	100	0·5	60

* Activity = daily dose in mg. per pigeon.

It will be noticed that 0·1 *N* NaOH is on the whole not destructive to vitamin B_1 in 10 minutes in the active preparation. In 1 hour complete inactivation takes place, whereas the growth factor is left untouched unless certain impurities are present.

The most active preparation tested (0·06 mg. curative) was not inactivated for *S. corallinus* by extensive alkali treatment.

These experiments definitely prove that the curative factor for the pigeon is not identical in all respects with the growth-promoting factor for *S. corallinus*. It is, however, interesting to note that the most active vitamin B_1 preparations still promote growth of *S. corallinus*. This effect is also quantitatively the same per pigeon dose as that of the preparations which are 100 or more times less pure. This suggests, as has occurred to others, that the same "nucleus" might be involved in each case, some slight change making the factor unavailable for the pigeon, though not for the bacillus. Admittedly, the only final evidence upon this point can be obtained by way of the pure substances. We have, however, tried to obtain indirect evidence by following step by step in the fractionations the two activities. In order to save space

we shall content ourselves with giving certain illustrative examples in the accompanying table (Table II), confining these to different types of fractionation.

Table II.

A. Silver precipitation from final alcohol fractionation (0·15 mg.).

Experiment	Total dosage S.T.	Total dosage P.T.
1. Original	1400	1400
2. 1st Ag ppt.	350	350
3. 2nd Ag ppt.	250	160
4. Filtrate	900	630
5. 1st Ag reppt. from (2)	200	350
6. Filtrate from (5)	100	

B. H_2PtCl_6 fractionation (XXX–XXXIII)

1. 1st H_2PtCl_6 ppt.	400–500	678
2. Filtrate	250	200
3. Reppt. H_2PtCl_6	200	140
4. Filtrate	50	—

C. Alcohol fractionation.

1. Original	600–800	600–800
2. 75 % alc. ppt.	4	Nil
3. Filtrate from (2)	600	600
4. 90 % alc. ppt.	550	550
5. Filtrate	24	Nil

Table II shows how closely the distribution of the two factors is followed in the fractions. Three cases of discrepancy have come to light. The so-called "anomalous" case has been discussed in the preceding communication. After a certain stage in the purification, it does not occur. The case of alkali has received mention in Table I. In certain cases of treatment with phosphotungstic acid, it was occasionally found that activity for the bacillus could be regenerated without a corresponding activity for the pigeon. This seems to be a parallel to the case of alkali previously mentioned, as the reverse has never occurred. We have attributed it to the combined action of alkali and phosphotungstic acid. Apart from these two results, not more than 10 % among the 133 parallel tests carried out during the last 18 months have been outside the limits of the experimental error of the test. These we cannot explain. We have excluded from this computation tests which gave "anomalous" results and those in which there was evidence of the toxic action of metals. There have also not been included a number of tests made upon material at intermediate stages of fractionation, which form a valuable body of indirect evidence as to the close parallelism of the two factors. Quite often it has been possible to predict the distribution of vitamin activity by the aid of the growth test. So if alkali treatment is avoided, the *S. corallinus* test has value in testing for vitamin B_1 after a certain stage. We feel that this is the strongest evidence which can be produced at present as to the close similarity of the two factors.

Discussion.

Our results confirm those of the Japanese workers in the following respects. A yeast vitamin B_1 preparation, just as their rice vitamin B_1 preparation, has bacterial growth-promoting power upon a *Streptothrix*; this organism therefore behaves similarly to their *Streptococcus haemolyticus*. The vitamin B_1 effect is readily destroyed by alkali, whereas the bacterial factor is less sensitive. In this respect the results differ, because our preparations which reached an activity considerably higher than those of the Japanese workers could be heated for 1 hour at 100° in $N/2$ NaOH solution without losing their growth-promoting effect.

It is interesting at this stage to combine the various data as to the action of what may be termed the "charcoal" concentrates. In addition to the facts presented in this paper, certain preliminary work has already been published by Peskett [1926]. He showed that the fraction of activity 0·08 mg. had bios activity. This remained upon heating with alkali, an operation which largely destroyed the vitamin B_1 effect. Further evidence as to the duality of bios and vitamin B_1 was obtained by Hawking [1927] and Peskett [1927]. *Saccharomyces cerevisiae* can synthesise vitamin B_1 from a medium containing small amounts of bios. In so far as the thermostable factor for rat growth, vitamin B_2, is not present in the charcoal concentrates, neither bios nor the *S. corallinus* factor can be identical with vitamin B_2.

Mention may also be made of the work of Heaton [1926], who has shown that the same concentrate of activity 0·08 mg. has growth-promoting effect upon epithelial tissue (in tissue cultures). Hence the thermostable vitamin B_2 does not seem to be required even for this growth, which is puzzling in view of its relation to the pellagra-like condition in rats.

In view of the results obtained by the Japanese workers it is interesting to note that two of us found that a strain of *Meningococcus* would grow upon a broth medium which had been completely freed from the *Streptothrix* factor and from vitamin B_1 by treatment with charcoal. Doubtless this depended upon the presence of one of the amino-acids mentioned by them. However, it was possible to show in extension of their results for *B. coli* that, after the growth of *Meningococcus*, there was present in the medium a growth-promoting factor for *S. corallinus*. Unfortunately, owing to the toxicity of the medium after the growth of *Meningococcus*, it has not been possible to show whether vitamin B_1 for the pigeon is also present. The facts for this organism are therefore also in accordance with the theory that those organisms which can grow upon a medium free from the factor for *S. corallinus* are able to synthesise it. It is clear that there is complexity among the lower organisms as regards their requirements in these factors [see Mueller, 1922], and that much work must be done before such a theory can be made general.

The work upon the relation of the curative factor in the charcoal concentrates to other factors is summarised in Table III.

Table III.

Stage of concentration	Activity mg.	Vitamin B: Curative B_1	Vitamin B: Thermostable* B_2	Bios† yeast growth-promoting	*Streptothrix* growth-promoting	Epithelial tissue‡ growth-promoting
Aqueous yeast extract	50 approx.	+	+	+	+§	+
Charcoal concentrates	0·5–1·0	+	Nil	+	+	+
Ag-baryta (previous exps.)	0·08	+	—	+	+	+
Phosphotungstic acid	0·1	+	—	—	+	—
H_2PtCl_6 in alcohol	0·027–0·06	+	—	—	+	—
Treatment with alkali	0·027–0·06	Nil	—	—	+	—
Treatment with alkali	0·08	Nil	—	+	+	—

* From Chick and Roscoe [1927].
† From Peakett [1926].
‡ From Heaton [1926].
§ Anomalous.
\+ Present.
— Not tested.

Conclusions.

1. Parallel tests upon the pigeon and *Streptothrix corallinus* have been made during the course of numerous fractionations of yeast concentrates.

2. Using the pigeon dose as a standard, the most active vitamin B_1 preparations (0·027 mg.) have the same relative growth-promoting powers as preparations 100 times less pure.

3. Sufficient treatment with alkali always inactivates the curative properties, but the purer extracts after such treatment still retain growth-promoting activity. From this it is concluded that vitamin B_1 and the bacterial growth-promoting factor are not identical.

4. Similarity in constitution is suggested by the fact that during varying types of fractionation (alcoholic, metallic precipitation, etc.) the two properties fractionate in parallel.

5. Since the *Streptothrix* factor is present in the charcoal concentrates, it is not identical with vitamin B_2.

Two of us (V. R. and H. W. K.) are indebted to the Medical Research Council for a grant.

Note by R. A. Peters.

In a paper upon the vitamin requirements of certain yeasts and bacteria, Funk and Dubin [1921] make the following statement: "Peters believed at first that protozoa can live and divide on purely inorganic material, but he found subsequently that they become smaller and smaller and appeared to live at the expense of their own protoplasm: he thinks therefore that addition of vitamins is necessary for proper growth." I do not know upon what statement of mine this is based, and cannot find any words in my published work upon the question which could be interpreted to mean this. The matter is at present under investigation.

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LX. THE INFLUENCE OF NORMAL AND CANCEROUS BLOOD-SERUM ON PANCREATIC LIPASE ACTION AND THE EFFECT OF IONIC AND COLLOIDAL LEAD.

By ROBERT FREDERICK CORRAN
and WILLIAM CUDMORE McCULLAGH LEWIS[1].

From the Muspratt Laboratory of Physical and Electro-Chemistry, University of Liverpool.

(*Received January 24th, 1928.*)

The "accelerating[2]" action of blood-serum and bile salts on pancreatic lipase has been observed by many authors (Pottevin [1903], Terroine [1914], Hamsik [1911,1915], Mellanby and Woolley [1914], and others). Perhaps the most notable contributions to the study of the action of various activators, of physiological importance, on lipase are those of Rosenheim and of Shaw-Mackenzie [Rosenheim, 1910; Rosenheim and Shaw-Mackenzie, 1910, 1, 2, 3; Shaw-Mackenzie, 1911, 1912, 1913, 1914], and as the methods and conclusions of these authors are closely connected with the work described in this paper, a very brief *résumé* of their conclusions is here given. Rosenheim [1910] found that the lipase contained in a glycerol extract of the pancreas could be separated into two portions, one portion consisting of inactive prolipase, insoluble in water and destroyed by heat, and the other consisting of a co-enzyme, soluble in water and thermostable. Bile salts and serum, according to Shaw-Mackenzie and Rosenheim, act as co-enzymes. They also found that a number of different compounds of varying structure can exert an augmentative action on lipase, for example, alcohol (in small quantity), sodium oleate, cholate and glycocholate, saponin and digitonin. Cholesterol, on the other hand, has a marked

[1] An investigation carried out on behalf of the Liverpool Medical Research Organisation; Prof. W. Blair Bell, director.

[2] "Acceleration" is the term apparently very generally employed in the literature of the subject, which deals with the influence exerted by various substances on the activity of lipase. Actually the greater part of the results recorded refer to changes in the *extent* of lipolysis effected, the values given being intended, in general, to represent final equilibrium values. Very frequently an increase in the extent of lipolysis is accompanied by an increase in the *rate* at the same time. But at least one case is recorded in the present paper where an increase in speed is accompanied by a reduction in the extent of lipolysis. Obviously the term "acceleration" ought to refer to alteration in rate, whilst the term "augmentation," *i.e.* augmentation of lipolysis, might be suggested as suitable for indicating an alteration in the extent (*i.e.* the final equilibrium values) of lipolysis. It is in this sense that the term "augmentation" is employed in the present paper.

diminishing action on the lipolytic augmentation of serum and bile salts. Concerning the nature of the co-enzyme of lipase present in serum no definite conclusions have been arrived at. Whereas cholesterol diminishes lipolytic action, lecithin derivatives augment it. Moreover, Willstätter [1923] has shown that simple salts of sodium and calcium can act as augmentors, consequently the inorganic salts play a part.

The results of Shaw-Mackenzie and Rosenheim show that in certain pathological conditions the augmentative action of serum is abnormal. They find that in human cases of scarlet fever and cancer the lipolytic augmentation is reduced and that, in cases of spontaneous recovery from cancer in mice, the augmentative action returns to normal. The results, however, do not appear to be conclusive, for in at least one paper [1910, 3] their results lead to the conclusion that cancer-serum augments to a much greater extent than does normal serum.

The work described in this paper was carried out to ascertain whether cancer-serum differed from normal serum with respect to its lipolytic co-enzyme content, and to study the effect of lead, in both the ionic and colloidal form, on the lipolytic augmentation produced by serum, both normal and cancerous.

Materials and method.

It was essential to use an enzyme extract which possessed the advantages of being reasonably pure and, at the same time, of being stable. An extract similar to that of Shaw-Mackenzie was employed. The method of preparation consisted of extraction of fresh, minced pig pancreas (which had been thoroughly washed with distilled water) with twice its weight of glycerol for 24 hours, and filtration of the mixture through a double layer of fine muslin. The turbid filtrate, containing lipase and also trypsin, proved to be a very satisfactory lipase extract in that the enzyme extract (kept in sterilised vessels in the ice-chest) was as active after a period of 9 months as at the beginning, thus differing from the enzyme extracts prepared by extraction with physiological salt solution [Amaki, 1926], by adsorption [Willstätter, 1923], or from pancreatic juice [Mellanby and Woolley, 1914], all of which were comparatively unstable.

As substrate an olive oil emulsion was used. The emulsions used by previous workers were generally prepared by neutralising the free fatty acid, present in the commercial oil, with standard sodium hydroxide, the soap formed acting as emulsifying agent.

As, however, different samples of olive oil contain varying amounts of free fatty acid, and consequently the composition of an emulsion prepared in the above manner varies, it was evident that an emulsion of pure olive oil with pure sodium oleate should be used to obtain a reliable substrate.

Pure olive oil was prepared by shaking the commercial oil with an equal volume of 10 % sodium hydroxide in a separating funnel at as high a temperature as the latter would stand (about 75°). After standing, the lower aqueous layer was separated and the emulsion of olive oil remaining washed with hot

distilled water until all soap was removed (in general about twelve washings were required). The olive oil was filtered through filter paper until all turbidity had disappeared and the oil dried finally by heating in an air-oven at 105° for 6 hours in a shallow basin. The oil was kept, pending use, in a sterilised flask fitted with a tight stopper in order to minimise the chance of production of free acid by bacterial action.

Pure sodium oleate was prepared by a modification of the method quoted by Leathes [1925]. Commercial olive oil was saponified with excess sodium hydroxide, the fatty acids were liberated by means of mineral acid and converted into lead salts by digestion on a water-bath with one-twentieth of their weight of litharge. Lead oleate was extracted with ether (the other lead soaps being insoluble in ether) and oleic acid liberated by the addition of dilute hydrochloric acid, the ether then being evaporated off. Neutralisation of the acid with standard aqueous sodium hydroxide in presence of alcohol gave a solution of sodium oleate, which could be adjusted, after removal of the alcohol, to the concentration required (1 % by weight).

In the experiments referred to hereafter, the enzyme extract, as described above, was used and an emulsion (prepared from four parts of pure olive oil and one part of 1 % sodium oleate solution) employed as substrate, except where otherwise stated. The emulsion used was fine grained, the size of the particles being on the average 5μ.

The experimental procedure was similar in principle to that of Shaw-Mackenzie and Rosenheim, that is, it consisted of mixing the requisite quantities of lipase extract, serum, water (or aqueous solution) and oil emulsion and allowing them to react, at a temperature of 37°, until equilibrium was attained[1]. The amount of hydrolysis was then determined by titrating the fatty acids produced against 0·1 *N* aqueous sodium hydroxide in presence of neutral alcohol (up to 40 % alcohol) and ether[2], using phenolphthalein as indicator.

I. Experiments with commercial olive oil emulsions.

At the outset, measurements were carried out following the conditions of Shaw-Mackenzie, that is by using a commercial oil emulsion and normal ox serum, preserved under toluene. In general, the results obtained were similar to those obtained by him.

(*a*) *Effect of order of mixing of the reactants.*

Shaw-Mackenzie found that, if the enzyme and oil emulsion were mixed first and serum added last, no lipolytic augmentation by serum was observed, the augmentative action of serum only manifesting itself when serum was

[1] As the lipase extract possesses both lipolytic and liposynthetic properties, hydrolysis does not go to completion.

[2] Shaw-Mackenzie titrated in presence of alcohol only. Experiment showed that a more satisfactory end-point could be obtained by the addition of ether, *i.e.* following the mode of titration employed by Willstätter.

mixed with the enzyme and the emulsion added last. Experiments were therefore carried out to ascertain the effect of order of mixing of the reactants. In Tables I (*a*) and II (*a*) below, the enzyme and serum were mixed first, and the emulsion added last. In Tables I (*b*) and II (*b*) the enzyme and emulsion were mixed first, and the serum added last. The duration of the experiments is not arbitrary but corresponds to the attainment of equilibrium.

Table I (*a*).

Time of reaction, 18 hours. Temperature 37°.

Lipase extract cc.	Ox serum cc.	Water cc.	Emulsion cc.	*N*/10 NaOH cc.	Augmentation *N*/10 NaOH cc.
1	0·0	5·0	5	1·15	—
1	0·5	4·5	5	18·97	17·82
1	1·0	4·0	5	27·45	26·30

Table I (*b*).

Lipase extract cc.	Emulsion cc.	Water cc.	Ox serum cc.	*N*/10 NaOH cc.	Augmentation *N*/10 NaOH cc.
1	5	5·0	0·0	1·00	—
1	5	4·5	0·5	20·27	19·27
1	5	4·0	1·0	16·48	15·48

Table II (*a*).

Glycerol extract diluted with twice its volume of water.

Lipase extract cc.	Ox serum cc.	Water cc.	Emulsion cc.	*N*/10 NaOH cc.	Augmentation *N*/10 NaOH cc.
1	0·0	5·0	3	0·33	—
1	0·5	4·5	3	10·89	10·56
1	1·0	4·0	3	17·86	17·53
1	2·0	3·0	3	19·20	18·87
1	3·0	2·0	3	20·75	20·42

Table II (*b*).

Lipase extract cc.	Emulsion cc.	Water cc.	Ox serum cc.	*N*/10 NaOH cc.	Augmentation *N*/10 NaOH cc.
1	3	5·0	0·0	0·30	—
1	3	4·5	0·5	15·50	15·20
1	3	4·0	1·0	19·70	19·40
1	3	3·0	2·0	16·95	16·65
1	3	2·0	3·0	16·45	16·15

It will be seen that in Tables I (*a*) and II (*a*) the effect of increasing concentration of serum is progressive up to a limit, but that in Tables I (*b*) and II (*b*) a maximum value is observed beyond which further addition of serum gives reduced values for the augmentative effect. It is of importance, therefore, that in carrying out comparative experiments, the reactants be mixed always in the same order. In the experiments described later, the serum and glycerol extract were mixed first, then the necessary quantity of water, or aqueous solution, and finally the oil emulsion. In the experiments with lead compounds, the serum was mixed first with the lead compound (a period of approximately

1 hour elapsing in order that equilibrium between the serum and the lead compound might be attained), then the glycerol extract of the enzyme was added followed by the water and the emulsion.

(*b*) *Effect of filtration of the glycerol extract on its lipolytic activity.*

Several workers have stated that, on filtration, the glycerol extract of lipase loses its activity. On the other hand, Hamsik found that filtration had no effect on the lipolytic activity of the enzyme extract. This point was investigated and it was found that filtration through asbestos or a Jena glass filter robbed the filtrate of its activity, the filter residues in both cases being highly active, but that filtration of the turbid extract through filter paper (type—Whatman No. 1) did not diminish the activity of the extract. Table III gives the results, using a turbid extract and a clear extract obtained by filtering the former through filter paper.

Table III

Lipase extract cc.	Ox serum cc.	Water cc.	Emulsion cc.	*N*/10 NaOH cc.	Augmentation *N*/10 NaOH cc.
Unfiltered turbid					
1	0	5	5	1·32	—
1	1	4	5	19·85	18·53
Clear paper-filtered					
1	0	5	5	1·38	—
1	1	4	5	20·89	19·51

It is probable, therefore, that the lipase occurs in the form of a finely divided suspension, rather than in the form of a coarse suspension.

II. Experiments with pure olive oil emulsions.

Experiments were next carried out using pure olive oil and pure sodium oleate, and the effect of normal human serum was investigated on different glycerol extracts (marked *A*, *B*, and *C* in Table IV), in order to find out if

Table IV.

Time of reaction 48 hours. Temperature 37°.

	Lipase extract cc.	Normal serum cc.	Source of serum	Water cc.	Emulsion cc.	*N*/10 NaOH cc.	Augmentation *N*/10 NaOH cc.
A	1	0	—	5	5	20·58	—
	1	1	R. F. C.	4	5	41·00	20·42
B	1	0	—	5	5	22·42	—
	1	1	J. W.	4	5	43·28	20·86
	1	0	—	5	5	22·00	—
	1	1	E. H.	4	5	41·65	19·65
C	1	0·0	—	5·0	5	22·61	—
	1	0·5	S. K.	4·5	5	41·30	18·69
	1	0·0	—	5·0	5	21·60	—
	1	0·5	H. H.	4·5	5	39·44	17·84
	1	0·0	—	5·0	5	21·70	—
	1	0·5	L. T.	4·5	5	39·93	18·23

reproducible results could be obtained. In Table IV the augmentations produced by different samples of normal human serum are given using the different extracts. The initials in column 3 refer to the different people from whom the blood was obtained.

In the first place, it is apparent that the different samples are comparable with one another, and that the various samples of blood-serum yield very consistent values for their lipolytic augmentations. In the second place, the time of reaction was 48 hours, experiment showing that equilibrium had then been attained. The emulsion of pure olive oil was considerably less stable than the commercial emulsion of Shaw-Mackenzie and it was found necessary to shake the reaction vessels during the course of the present work at intervals of 10–12 hours in order to ensure the maintenance of complete emulsification of the oil[1]. In the third place, the magnitudes of the control readings are considerably greater than those of Shaw-Mackenzie, who obtained large control readings in only a few cases. In many other cases, the control readings observed by Shaw-Mackenzie were less than 5 cc. As the principal uncontrolled variable in Shaw-Mackenzie's procedure was the amount of sodium oleate present, the influence of this salt on lipase action was investigated.

The rôle of sodium oleate in esterase and lipase action.

Three different substrates were used, (*a*) methyl acetate (a typical substrate for ester hydrolysis), (*b*) triacetin, as an example of a soluble glyceride, (*c*) pure olive oil. In the experiments with (*a*) and (*b*) a 10 % solution of the substrate in water was used. Tables V–VII give the mean results obtained on the influence of varying quantities of a 1 % aqueous solution of sodium oleate on esterase and lipase action. The values recorded are in all cases the mean of at least two (concordant) sets of experiments.

Table V.

Temperature 37°.

Lipase extract cc.	Water cc.	1 % sodium oleate solution cc.	10 % aqueous methyl acetate cc.	Time hrs.	*N*/10 NaOH cc.
1	5·00	0·00	5	48	2·73
1	4·75	0·25	5	48	2·79
1	4·50	0·50	5	48	2·92
1	4·25	0·75	5	48	2·98
1	4·00	1·00	5	48	2·82
1	3·50	1·50	5	48	3·00
1	3·00	2·00	5	48	3·03
1	0·00	5·00	5	48	3·05

It is clear from Table V that the esterase content of the glycerol extract employed is very small, and no conclusions can be drawn from the results. On the hydrolysis of triacetin, sodium oleate exerts a marked augmentative action up to a definite concentration beyond which the augmentation dimin-

[1] This was tested from time to time by microscopic measurement of the size of the oil droplets.

ishes. The influence of sodium oleate on olive oil hydrolysis is remarkable in that, with increasing concentration of sodium oleate, a maximum degree of lipolysis, *i.e.* maximum augmentation, is reached beyond which the effect diminishes rapidly, so that in high concentration sodium oleate exerts a diminishing action on the *extent* of olive oil hydrolysis. On the other hand, sodium oleate, in increasing concentration, causes a progressively increasing acceleration of hydrolysis. In all the experiments recorded in Table VII, the system was repeatedly shaken. With low concentrations of oleate only temporary emulsification was obtained. With quantities of oleate greater than 1 cc. the emulsification was permanent.

Table VI.

Temperature 37°

Lipase extract cc.	Water cc.	1 % sodium oleate solution cc.	10 % aqueous triacetin cc.	Time hrs.	*N*/10 NaOH cc.	Augmentation *N*/10 NaOH cc.
1	5·0	0·0	10	48	8·86	—
1	4·5	0·5	10	48	9·95	1·09
1	4·0	1·0	10	48	10·34	1·48
1	3·0	2·0	10	48	10·69	1·83
1	0·0	5·0	10	48	9·70	0·84

Table VII.

Temperature 37°

Lipase extract cc.	Water cc.	1 % aqueous sodium oleate cc.	Olive oil cc.	Time days	*N*/10 NaOH cc.	Augmentation *N*/10 NaOH cc.
1	6·00	0·00	4	10	9·81	—
1	5·75	0·25	4	8	12·12	+ 2·31
1	5·50	0·50	4	6	14·98	+ 5·17
1	5·25	0·75	4	4	20·80	+10·99
1	5·00	1·00	4	3	22·68	+12·87
1	4·50	1·50	4	2	29·23	+19·42
1	4·00	2·00	4	2	35·80	+25·99
1	1·00	5·00	4	2	5·57	− 3·24
1	1·00	5·00*	4	2	2·80	− 7·01

* Of 5 % sodium oleate.

In Shaw-Mackenzie's experiments it was found that 5 cc. of commercial olive oil required, in general, 1–2 cc. of *N*/10 NaOH to neutralise the free acid present. Considering the two extreme values, namely 1 cc. and 2 cc. *N*/10 NaOH, calculation shows that 5 cc. of an olive oil emulsion, prepared by using 1 cc. and 2 cc. of *N*/10 NaOH respectively for neutralisation, contain the equivalent of 2·4 cc. and 4·3 cc. of 1 % aqueous sodium oleate, that is, a concentration region beyond the maximum value given in Table VII. Moreover, the greater the amount of free fatty acid contained in the commercial olive oil, the smaller will be the value of the control reading. It is probable therefore that variation in sodium oleate content is the chief cause of the variability observed in the control readings in Shaw-Mackenzie's results.

III. Comparison of the lipolytic augmentations of normal and cancerous serum.

The samples of blood were taken from the forearm and were, in all cases, centrifuged as soon as possible after extraction from the patient in order to avoid any change in the constitution of the serum. The reaction vessels consisted of thick-walled glass bottles of 100 cc. capacity, fitted with ground-glass stoppers; the bottles were subsequently used as titration vessels. The mode of following the reaction was the same as that described previously. In all the experiments carried out 1 cc. of enzyme extract was mixed with 0·5 cc. (or 1 cc.) of serum and the mixture made up to 6 cc. in every case by the addition of water. 5 cc. of the pure olive oil emulsion, previously mentioned, were then added and the reaction allowed to proceed. The following Tables VIII–X give the results obtained with normal and cancerous human serum. Under the column headed "source of serum" are given numerals, which serve to identify the different samples of serum used. In Table X a summary is given of the patients from whom blood was extracted, corresponding to the various numerals.

Table VIII.

Time of reaction 48 hours. Temperature 37°

Amount of serum used cc.	Source of serum	Augmentation by serum *N*/10 NaOH cc.
1·0	Normal	**20·42**
1·0	I	18·34
1·0	Normal	**20·86**
1·0	II	14·10
1·0	III	14·88
1·0	Normal	**19·65**
1·0	VIII	12·30
0·5	Normal	**17·56**
0·5	IV	11·44
0·5	V	12·52
0·5	Normal	**17·84**
0·5	IX	17·24
0·5	X	11·20
0·5	Normal	**18·64**
0·5	VI	14·76
0·5	VII	12·53

Table IX.

Amount of serum used cc.	Source of serum	Augmentation by serum *N*/10 NaOH cc.
0·5	XII	12·93
0·5	XV	10·70
0·5	XVI	19·80
0·5	XVIII	15·30
0·5	XIX	10·64
0·5	XX	12·39
0·5	XXI	10·15
0·5	XXII	13·86
0·5	XXIII	13·60
0·5	XXIV	12·95
0·5	XXV	12·70

Table X.

Source of serum	Sex of patient	Initials	Nature of growth
I	Female	E. B.	Carcinoma cervix
II	Male	D. R.	Epithelioma tonsil
III	,,	J. H.	,, ,,
IV	Female	L. L.	Carcinoma cervix
V	Male	J. W.	Carcinoma tongue
VI	,,	J. M.	,, ,,
VII	,,	G. W.	Epithelioma tongue
VIII	Female	E. W.	Carcinoma stomach
IX	,,	E. C.	Sec. carcinoma chest
X	Male	J. B.	Epithelioma scrotum
XI	Female	E. S.	Carcinoma cervix
XV	,,	J. I.	Rec. carcinoma cervix
XVI	Male	H. A.	Carcinoma oesophagus
XVIII	Female	A. J.	Rec. carcinoma chest
XIX	Male	E. W.	Epithelioma pharynx
XX	Female	A. G. M.	Carcinoma stomach
XXI	,,	E. G.	Sec. carcinoma breast
XXII	,,	S. D.	Carcinoma breast
XXIII	,,	H. C.	Sec. carcinoma spine
XXIV	,,	L. W.	Carcinoma cervix
XXV	,,	M. J. B.	Post. nasal epithelioma

It is evident from the tables that there is a real abnormality in cancer-serum, in that it contains less of the lipolytic co-enzyme than does normal serum. This confirms the conclusions of Shaw-Mackenzie and Rosenheim. It will be observed that the deviations in some cases are greater than in others, even when the site of the tumour is the same. Inspection of Tables VIII–X demonstrates that in a few of the cases of malignancy investigated (cases I, IX, XVI), little difference exists in the augmentation by cancerous serum and normal serum. In fact, the sample of serum XVI augments lipase to a greater extent than does normal serum. It is significant that in this case the condition of malignancy was accompanied by secondary infections of the liver, infections of which are known to produce an excess of bile in the body, bile salts being active co-enzymes. In the other cases, however (I and IX), such secondary infections were not apparent.

It may be stated at this point that experiments were carried out in which the system was brought to a known initial p_H by means of a buffer (the ammonia-hydrochloric acid mixture of Michaelis and Willstätter) and the lipolytic augmentation of normal and cancerous serum investigated. The results obtained in these cases, though less exact owing to increased experimental difficulties (*e.g.* de-emulsification by the buffer), serve to bear out, in all respects, the conclusions arrived at above.

IV. The effect of ionic and colloidal lead compounds on the lipolytic augmentation of serum.

Having established a definite variation in the lipolytic co-enzyme content of cancerous and normal serum, the effect of lead, in a variety of forms, on the augmentation of lipase by serum has been investigated. In this connection, the following lead compounds have been used: (*a*) saturated aqueous lead

phosphate, $Pb_3(PO_4)_2$; (*b*) 0·02 *N* lead acetate solution; (*c*) a finely divided lead uspension—"S_7"—(used locally as a therapeutic agent) containing 0·5 g. lead per 100 cc.; (*d*) a colloidal suspension of lead sulphide containing 0·2 g. lead per 100 cc.; (*e*) colloidal lead selenide containing 0·2 g. lead per 100 cc.

The serum was mixed first with the lead compound, the enzyme extract added, followed by water and lastly the emulsion. In the experiments recorded, the effect of lead on the augmentation produced by normal ox serum and cancerous human serum has been investigated. Tables XI–XIV give the results obtained using the various lead compounds mentioned. In all cases the temperature is 37° and the duration 48 hours.

Table XI. *The effect of lead on the lipolytic augmentation of ox serum.*

Lipase extract cc.	Serum cc.	H_2O cc.	0·02 *N* Pb acetate cc.	Saturated Pb phosphate cc.	Lead suspension S_7 cc.	Colloidal PbSe cc.	Colloidal PbS cc.	Emulsion cc.	*N*/10 NaOH cc.
1	0·0	5·0	0	0	0	0	0	5	21·38
1	0·5	4·5	0	0	0	0	0	5	42·10
1	0·5	4·0	0·5	0	0	0	0	5	58·94
1	0·5	4·0	0	0·5	0	0	0	5	49·45
1	0·5	4·0	0	0	0·5	0	0	5	55·55
1	0·5	4·0	0	0	0	0·5	0	5	44·30
1	0·5	4·0	0	0	0	0	0·5	5	54·16

Table XII. *The effect of lead on human cancerous serum. Serum IV.*

Lipase xtract cc.	Serum cc.	H_2O cc.	0·02 *N* Pb acetate cc.	Saturated Pb phosphate cc.	Lead suspension S_7 cc.	Colloidal PbSe cc.	Colloidal PbS cc.	Emulsion cc.	*N*/10 NaOH cc.
1	0·0	5·0	0	0	0	0	0	5	21·70
1	0·5	4·5	0	0	0	0	0	5	32·60
1	0·5	4·0	1	0	0	0	0	5	39·30
1	0·5	2·5	0	2	0	0	0	5	43·80
1	0·5	3·5	0	0	1	0	0	5	43·36
1	0·5	3·5	0	0	0	1	0	5	38·28
1	0·5	3·5	0	0	0	0	1	5	52·18

Table XIII. *Serum V.*

Lipase extract cc.	Serum cc.	H_2O cc.	0·02 *N* Pb acetate cc.	Saturated Pb phosphate cc.	Lead suspension S_7 cc.	Colloidal PbSe cc.	Colloidal PbS cc.	Emulsion cc.	*N*/10 NaOH cc.
1	0·0	5·0	0	0	0	0	0	5	21·20
1	0·5	4·5	0	0	0	0	0	5	33·68
1	0·5	4·0	0·5	0	0	0	0	5	40·40
1	0·5	4·0	0	0·5	0	0	0	5	37·20
1	0·5	4·0	0	0	0·5	0	0	5	45·20
1	0·5	4·0	0	0	0	0·5	0	5	42·16
1	0·5	4·0	0	0	0	0	0·5	5	45·36

Table XIV. *Serum XV.*

Lipase extract cc.	Serum cc.	H_2O cc.	0·02 *N* Pb acetate cc.	Saturated Pb phosphate cc.	Lead suspension S_7 cc.	Colloidal PbSe cc.	Colloidal PbS cc.	Emulsion cc.	*N*/10 NaOH cc.
1	0·0	5·0	0	0	0	0	0	5	21·40
1	0·5	4·5	0	0	0	0	0	5	32·45
1	0·5	4·0	0·5	0	0	0	0	5	39·57
1	0·5	4·0	0	0·5	0	0	0	5	36·00
1	0·5	4·0	0	0	0·5	0	0	5	41·00
1	0·5	4·0	0	0	0	0·5	0	5	34·95
1	0·5	4·0	0	0	0	0	0·5	5	37·50

It is evident from the preceding tables that lead, in both the ionic and colloidal forms, exerts a definite augmentative action on the lipolytic augmentation of serum. Although the influence of lead on the lipolytic augmentation of serum appears to be established, the magnitude of the action varies considerably from experiment to experiment. It may be pointed out, moreover, that these effects, although provisionally attributed to lead, might possibly not be specific to that metal; they may have their origin, in part at least, in the precipitation and removal from the field of some anti-enzymic material, possibly of a protein nature.

The effect of ionic and colloidal lead in low concentration.

In the experiments recorded above the concentrations of lead employed were high compared to that of the serum, the addition of the lead acetate solution, colloidal lead (S_7) and the suspension of lead sulphide causing precipitation of the greater part of the carbonate and phosphate: the lead selenide and lead phosphate solution cause no precipitation. The effect of lead in amounts insufficient to precipitate any of the constituents of serum is of special interest.

10 cc. of clear, fresh, ox serum were treated with 0·2 cc. of the various lead compounds employed in the last section for 6 hours. With the exception of lead selenide, it was found that clear mixtures remained, the dark colour of the lead suspensions having disappeared. In the case of the selenide, the dark colour of the latter remained after standing for 6 hours. 0·5 cc. of the mixture in each case was used. As a control 10 cc. of ox serum were mixed with 0·2 cc. of water and 0·5 cc. of the mixture used. The results obtained are given in Table XV.

It is clear that lead, in very low concentration, can increase the lipolytic augmentation produced by serum. Moreover, the results of Table XV lead to the conclusion that the effect of lead is due to interaction of lead with some constituent of the serum. It may be pointed out that the effect of lead is still apparent when the latter is present in quantity sufficient to precipitate the greater part of the phosphate and carbonate of the serum. It may also be mentioned that the augmentation obtained with the small quantities of lead

compounds (Table XV) are of the same order of magnitude as those obtained with much greater concentrations of lead. Thus, taking the case of saturated lead phosphate, the concentration in Table XV is one-fiftieth of that employed in Table XII, whilst the augmentation due to the smaller concentration is more than one-half that obtained with the greater concentration.

Table XV.

Lipase extract cc.	Serum cc.	Identification of serum	H_2O cc.	$N/10$ NaOH cc.	Augmentation $N/10$ NaOH cc.
1	0·0	—	5·0	20·00	—
1	0·5	*A*	4·5	41·00	21·00
1	0·5	*B*	4·5	46·30	26·30
1	0·5	*C*	4·5	43·90	23·90
1	0·5	*D*	4·5	48·30	28·30
1	0·5	*E*	4·5	42·70	22·70
1	0·5	*F*	4·5	48·30	28·30

A = mixture consisting of 10 cc. ox serum + 0·2 cc. H_2O
B = " " 10 cc. " + 0·2 cc. 0·02 *N* lead acetate
C = " " 10 cc. " + 0·2 cc. saturated lead phosphate
D = " " 10 cc. " + 0·2 cc. colloidal lead (S_7)
E = " " 10 cc. " + 0·2 cc. colloidal lead selenide
F = " " 10 cc. " + 0·2 cc. colloidal lead sulphide

Discussion.

Considering the results collectively, it is clear that they afford a satisfactory confirmation of the conclusions arrived at by Shaw-Mackenzie and Rosenheim regarding the real augmentation of lipase action by normal serum and the reduced, but still positive, effect produced by the sera of cancer patients. The present data, it is believed, afford a more reliable basis for this conclusion than those given by Shaw-Mackenzie. The entirely new result is obtained that lead in a variety of forms—ionic and colloidal—when added to serum, either normal or cancerous, produces an increase in the augmentative effect. The presence of lead opposes and annuls the depression in lipolytic augmentation produced by cancerous serum as compared with normal serum.

Of the nature of the co-enzyme in serum we know as yet very little. Rosenheim has considered that it is hormonal in nature but existing evidence is insufficient to justify any definite conclusion.

Summary.

1. The previous work on the influence of serum on pancreatic lipase is described briefly.

2. The method of preparation of the reactants is outlined. Experiments carried out using a commercial olive oil emulsion showed that (*a*) variation in the order of mixing of the reactants gave quantitatively different results for the effect of ox serum on lipase action, (*b*) filtration, through filter paper, has no effect on the activity of the glycerol extract of lipase.

3. A series of experiments has been carried out using purified olive oil and known quantities of sodium oleate as emulsifying agent. It has been found

that in concentrations up to 0·2 g. oleate per 100 cc. of the mixture, this salt acts, on the *extent* of fat hydrolysis, as an augmentor, whilst beyond this concentration the augmentative effect diminishes and with a concentration of 0·5 g. of oleate per 100 cc. mixture the effect is less than in the absence of the oleate. On the *rate* of hydrolysis, sodium oleate acts as an accelerator in all concentrations employed.

Sodium oleate likewise acts as an augmentor for the hydrolysis of triacetin, which forms a solution in water, the augmentation rising to a maximum at a concentration of 0·3 g. oleate per 100 cc. Thereafter, the augmentation diminishes slightly. It is concluded therefore that the oleate, as a co-enzyme, functions by interaction with the lipase and not with the surface of the substrate, although, when the latter is insoluble, the oleate is a highly efficient emulsifier.

4. Experiments on the comparative actions of normal and cancerous human serum on olive oil hydrolysis (*a*) in systems in which no attempt was made to control the initial p_H of the system, (*b*) in systems brought to a known initial p_H by means of a buffer, show that cancer-serum has a reduced augmentative effect on lipase, as compared with normal serum, thus confirming the conclusions of Shaw-Mackenzie.

5. The effect of ionic and colloidal lead on the lipolytic augmentation of serum has been investigated and it is found that lead, in both the ionic and colloidal forms, increases the lipolytic augmentation of both normal and cancerous sera. This effect manifests itself whether the lead compound is present in amounts either sufficient or insufficient to cause a precipitate with serum.

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LXI. A COMPARISON OF THE EFFECTS OF GLUCOSE AND DIHYDROXYACETONE ON METABOLISM.

By MAURICE WALTER GOLDBLATT (*Beit Memorial Research Fellow*).

From the Medical Unit, St Thomas's Hospital, London.

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The question as to whether dihydroxyacetone is or is not directly oxidised in the animal body is one which is being keenly debated. Campbell and Markowitz [1927], working with depancreatised dogs, found that the triose administered to such animals is excreted quantitatively as glucose and is not metabolised in the absence of insulin. Insulin, however, is not necessary for its conversion to glucose. Further, in these experiments the R.Q. was not raised and ketosis was not prevented even when insulin was administered. These authors conclude that there is no evidence that dihydroxyacetone is an intermediary compound in the metabolism of glucose.

Embden, Schmidt and Wittenberg [1913] found that glucose is formed when the surviving liver of the dog is perfused with blood containing dihydroxyacetone. It has also been shown that this substance can give rise to glycogen. Markowitz and Campbell [1926] conclude that it must be transformed to glucose in the liver before it can be metabolised. It has further been shown that it can overcome the hypoglycaemia following an excessive dose of insulin [Campbell and Hepburn, 1926; Kermack, Lambie and Slater, 1926]. The fact, however, that this triose brings about, both in animals and man, a much more rapid and much greater rise in the respiratory metabolism than an equivalent dose of glucose, made it probable that it can be, at least in part, directly oxidised without previous transformation into glucose [Mason, 1926; Lambie and Redhead, 1927].

Cathcart and Markowitz [1927, 1] compared the effects of glucose, sucrose, laevulose, galactose, maltose, lactose and dihydroxyacetone on the respiratory metabolism in man, and found, in agreement with Higgins [1916] and others, that laevulose, galactose and sucrose cause a sharper and higher rise in R.Q. than glucose. They also confirm Mason's findings with dihydroxyacetone that the R.Q. following this substance exceeds unity. They then argue that, since dihydroxyacetone cannot be used directly but must first be transformed to glucose, these results cannot be interpreted as indicating preferential oxidation. They suggest that the conception of the respiratory quotient as a combustion quotient must be revised and that it represents rather the algebraic sum of

the transformations carbohydrate $\rightleftharpoons$ fat plus the oxidation of carbohydrate for energy purposes. The direction of the reaction carbohydrate $\rightleftharpoons$ fat is conceived as dependent on the "tension" of carbohydrate in the liver; thus, when this tension is so high that gluco-neogenesis from fat and protein is no longer necessary, one obtains a respiratory quotient greater than unity and fat is formed from the sugar.

It is not, however, at all obvious why certain of the sugars normally foreign to the blood should give us a high carbohydrate tension and hence a R.Q. greater than unity, whilst glucose does not do this. Cathcart and Markowitz suggest that when dealing with sugars foreign to the organism the liver rapidly removes them from the circulation and so brings about a great rise in carbohydrate tension.

As judged, however, by glycogen determinations in the liver and muscles of animals following the administration of the various sugars, one is led to conclude that glucose is the sugar *par excellence* for increasing carbohydrate tension. To take the case of glucose and galactose, the administration of 50 g. of these sugars to rabbits weighing 2 kg. will, in the case of glucose, produce about 10 % of glycogen in the liver, whilst in that of galactose only about 2·5 % is obtained, 4 or 5 g. of galactose being found in the urine.

Further, glucose is certainly the best of all sugars for combating ketosis induced by whatever means, and it seems fairly certain that this antiketogenic action is due to a deflection of metabolism from a preponderance of fat to a preponderance of carbohydrate. Thus the effectiveness of a sugar as an antiketogenic agent would be proportional to the carbohydrate tension which it could bring about in the liver and tissues. On Cathcart's view we would expect the best antiketogenic sugars to be those which produce the greatest rise in R.Q., but in point of fact this is not the case, galactose, sucrose and laevulose being less effective in this respect than glucose, in spite of the fact that the former sugars all give higher R.Q.s than the latter. We shall see that dihydroxyacetone also is not very effective in the matter of antiketogenesis.

In the case of dihydroxyacetone the rise in R.Q. is so greatly over unity that it becomes necessary to suppose a considerable synthesis of fat to account for it.

Now it seemed to us that a method of testing certain aspects of the problem was presented by the effect of the administration of the triose to the starving human subject. In such a subject, after a period of about 2 days without food, a condition is attained in which the carbohydrate tension of Cathcart and Markowitz [1927, 2] is considerably lowered and the organism is thrown back on to its protein and fat for its carbohydrate and energy. The occurrence of a ketosis in these conditions indicates the preponderance of what may be called the fat type of metabolism [Raper and Smith, 1926] and the administration of any substance throwing extra fat on the metabolism is accompanied by an increase in the excretion of the ketonic acids. This is well shown in the following experiment.

Exp. 1. The writer was the subject of experiment. After 37 hours of complete starvation 50 g. of melted butter were ingested. The urine was collected at the times given in the table and the ketonic acids estimated by a modification of the Shaffer-Marriot method.

		Acetoacetic acid as mg. acetone		β-Hydroxybutyric acid mg.	
Time	Urine cc.	Total	Per hr.	Total	Per hr.
8.0 a.m.	270	17·2	1·4	45·2	3·8
9.0	Butter taken				
10.0	25	7·6	7·6	10·4	10·4
11.15	22	8·0	6·4	8·3	6·6
12.00	16	6·5	8·6	7·1	9·5
1.0 p.m.	22	8·7	8·7	8·1	8·1
2.10	23	11·4	9·8	14·8	12·7
3.30	24	13·8	10·3	18·4	13·8
4.30	18	12·7	12·7	19·5	19·5
6.0	31	24·1	16·1	40·9	27·3
6.30	15	10·5	21·0	28·2	56·4

These figures leave no doubt at all that at least the fatty acids found in butter readily bring about a large increase in the excretion of ketones in these conditions. Now in the matter of dihydroxyacetone it was argued that if it were all converted to glucose before being metabolised it should be at least as effective as glucose as an antiketogenic agent. If, on the other hand, it is to any great extent converted to fat, it should be demonstrable as a ketogenic substance: and, lastly, if both oxidation and transformation to fat are proceeding, its effect should be intermediate between ketogenesis and ketolysis.

In a previous communication [Goldblatt, 1925] it was shown that, in the case of the writer, after a period of about 40 hours' complete starvation 50 g. of glucose readjusted the keto-antiketogenic balance in 1 hour and no further excretion of ketonic acids occurred for 3½ hours more. It was further demonstrated that the ketosis was accompanied by a marked loss of tolerance to sugar, high blood-sugar curves and glycosuria occurring after doses of glucose normally easily tolerated. It was also found that the R.Q. in these conditions rose only very slightly compared to the findings in the usual post-absorptive state.

In the following experiments the effects of dihydroxyacetone are compared with those of glucose in similar conditions. The respiratory metabolism was determined by the Douglas-Haldane method after a period of complete rest of about 1 hour. The blood-sugar was estimated by MacLean's method. With regard to the effect of any circulating dihydroxyacetone on the blood-sugar as estimated by this method, it is rather surprising that the effect is in fact negligible, in spite of its powerful reducing properties. If, instead of using the usual 0·2 cc. of blood, one employs 0·2 cc. of a solution containing 0·1 % of dihydroxyacetone, the difference in the thiosulphate values of the alkaline copper solution before and after reduction does not exceed 0·10 cc. *N*/400. This seems to be due to the degree of alkalinity of the copper reagent, for considerably greater reduction values are obtained if a few drops of strong alkali are added.

The writer was the subject of experiment, except where otherwise stated.

Exp. 2. The effect of 50 g. glucose 16 hours after food. The sugar was taken immediately after the first R.Q.

Time mins.	Blood-sugar g. %	O_2 cc. per min.	CO_2 cc. per min.	Rise in O_2 intake %	R.Q.
0	0·102	177	135	—	0·76
30	0·150	189	155	6·8	0·82
60	0·113	187	164	5·6	0·88
90	0·091	176	159	—	0·90
120	0·075	149	120	—	0·81

Exp. 3. The effect of 50 g. glucose 20 hours after food.

0	0·081	212	167	—	0·79
30	0·146	220	169	3·8	0·77
60	0·156	226	185	6·6	0·82
90	0·122	210	176	—	0·84
120	0·100	184	159	—	0·86

Exp. 4. The effect of 50 g. glucose 41 hours after food.

Time mins.	Blood-sugar g. %	O_2 cc. per min.	CO_2 cc. per min.	Rise in O_2 intake %	R.Q.	Acetoacetic acid in urine as mg. acetone per hr.
0	0·113	241	176	—	0·73	5·7
25	0·104	238	176	—	0·74	6·1
40	0·184	—	—	—	—	—
55	0·189	264	198	9·5	0·75	5·6
70	0·187	—	—	—	—	0·0
85	0·189	256	207	6·2	0·81	0
105	0·141	—	—	—	—	0
215	0·099	—	—	—	—	0
265	—	—	—	—	—	2·3

Exp. 5. The effect of 50 g. dihydroxyacetone 15 hours after food.

Time mins.	Blood-sugar g. %	O_2 cc. per min.	CO_2 cc. per min.	Rise in O_2 intake %	R.Q.
0	0·100	197	170	—	0·86
30	0·097	234	292	18·8	1·25
60	0·067	221	195	12·2	0·88
90	0·081	230	182	16·8	0·79
120	0·095	231	164	17·3	0·71
150	0·077	—	—	—	—

Exp. 6. The effect of 50 g. dihydroxyacetone 40 hours after food.

Time mins.	Blood-sugar g. %	O_2 cc. per min.	CO_2 cc. per min.	Rise in O_2 intake %	R.Q.	Acetone bodies in urine as mg. acetone per hour: Aceto-acetic acid	Acetone bodies in urine as mg. acetone per hour: β-Hydroxy-butyric acid
0	0·072	208	160	—	0·77	13·14	9·72
15	0·072	—	—	—	—	25·96	6·04
25	—	—	—	—	—	—	—
35	0·095	275	249	32·2	0·903	23·11	3·16
45	—	—	—	—	—	—	—
55	—	—	—	—	—	9·60	7·74
60	0·095	253	222	21·6	0·88	—	—
87	—	—	—	—	—	—	—
95	0·095	228	162	9·6	0·71	1·56	2·27
115	—	—	—	—	—	—	—
125	0·077	223	164	7·2	0·74	2·41	6·84
150	0·056	—	—	—	—	3·90	3·90
215	—	—	—	—	—	3·56	2·58

Exps. 2, 3 and 4 show the diminishing tolerance to glucose which occurs as the starvation period is increased. The R.Q. shows definitely less rise, following the administration of the sugar, the longer the starvation, the maxima being 0·90 in Exp. 2, 0·86 in Exp. 3 and 0·81 in Exp. 4. The maximum rises in the oxygen intake in the successive cases were 6·8 %, 6·6 % and 9·5 %. Thus we cannot suppose that the lower R.Q.s found after starvation are to be interpreted as indicating diminished oxidation. The truth seems to be rather that the relative depletion of glycogen in the liver and the muscles brings about the necessity to attack administered carbohydrates as quickly as possible for energy purposes; so that the R.Q. in this case, on Cathcart's view given above, does not rise as high as ordinarily through the masking effect of simultaneous gluco-neogenesis from fat or protein or both. With such considerable increases in oxygen absorption as, for example, occur in Exp. 4 there is no question that oxidation has increased, so that if the R.Q., as at present usually interpreted, does not indicate increased oxidation, we can only conclude that the interpretation must be modified on some such lines as those laid down by Cathcart.

The high and prolonged blood-sugar curve obtained after starvation must mean that an effort is being made to satisfy the needs of the muscles for carbohydrate. As the transference of blood-sugar from the liver to the tissues is progressing, oxidation is going on simultaneously, a large increase in oxygen consumption occurring at once, but the maximum rise in R.Q. occurring when gluco-neogenesis is at a minimum. Thus we expect high R.Q.s during the fall in blood-sugar when the tissues are supplied with an effective head or tension of carbohydrate. This is in fact what occurs, as was found by Bornstein and Holm [1922] and Goldblatt [1925].

If this analysis of our findings in starvation is correct then the main disturbance in carbohydrate metabolism in that condition is a diminished power of storage in the liver, a condition which is determined by the carbohydrate depletion of the muscles. It seems, further, probable that when the carbohydrate tension of the tissues is lowered, there is a compensatory diminished activity on the part of the pancreas. Thus when highly fatty diets are fed, sufficient to produce ketosis, abnormally high and delayed blood-sugar curves are obtained [Kohn, Fries and Felshin, 1927]. On the above view, such diets would lead to a struggle for carbohydrate on the part of the muscles with a compensatory diminished secretion of insulin, thus delaying the fall in blood-sugar after a dose of glucose, until such time as the tissue-sugar were re-established.

Recent work by Grafe and Meythaler [1927] and Dietrich [1927] makes it very probable that glucose acts as a direct stimulant to insulin secretion, which would corroborate the view that after a period of starvation the response of the islet mechanism is more sluggish than under ordinary post-absorptive conditions.

We must now examine Exps. 5 and 6 to see if any similar results are obtained with dihydroxyacetone.

In Exp. 5, 15 hours after food, 50 g. of the triose produced hypoglycaemia in 1 hour, then a rise occurred in the blood-sugar and 2½ hours after the solution was taken there was again a tendency to hypoglycaemia. At no time did there occur a rise above the fasting blood-sugar level. These findings confirm those of Cathcart and Markowitz [1927, 2] and others.

This sort of effect can be obtained with less than 50 g. and in our experience much more rapidly if the triose be taken about 5 hours after a meal. The following figures give the blood-sugar findings, in the case of the writer, following 30 g. of dihydroxyacetone taken 5 hours after food.

Time p.m.	2.00	2.10	2.25	2.40	3.10	3.50
Blood-sugar g. %	0·097	Triose taken	0·109	0·067	0·083	0·104

If we regard this rapid removal of sugar from the blood as the result of a stimulus to the insulin-secreting mechanism, then it appears that even after 15 hours' fasting this stimulus has become less effective. If now we examine the blood-sugar values in Exp. 6, it is seen that for 120 minutes they are consistently above the fasting level and hypoglycaemia only occurs 150 minutes after the triose was taken. Here, then, there is a definite loss in the rapidity of response to the stimulus which dihydroxyacetone undoubtedly exerts on the mechanism which removes sugar from the blood.

In addition to this the R.Q. in Exp. 6 rose to 0·903, whilst in Exp. 5 it reached 1·25. In each case a large increase in oxygen utilisation occurred, in Exp. 5 amounting to a maximum of 18·8 % and in Exp. 6 to 32·2 %. It is thus again made clear that the starving organism shows much less tendency to form fat, at least as judged by the R.Q. But what is the function of the large increase in oxygen intake? There is no question of overbreathing, for this would become more manifest in the starving condition, when the subject is already somewhat acidosed, whereas the R.Q. is much lower in this case. Whatever interpretation be adopted for the R.Q. an increased intake of oxygen can only mean increased oxidation or conversion of fat or protein to carbohydrate. It is true that this increased oxygen intake is not of long duration, but this must, it seems to us, be regarded as due to a readjustment to the metabolism of a different proportion of fat, protein and sugar with the same oxygen need as was necessary in basal conditions, gluco-neogenesis being at a minimum, whilst carbohydrate is both being oxidised and changed to fat. The fact that we can never account for more than a small fraction of administered carbohydrate by the rise in oxygen consumption must mean that other reactions are occurring which mask the oxidation of the sugar.

This difficulty in interpreting the significance of the large though temporary increase in oxygen utilisation is occasionally absent in certain pathological cases which give R.Q.s exceeding unity even with glucose. In these cases there occurs a steady fall in oxygen intake clearly shown in the following two examples. In each case the analysis was carried out 15 hours after food, the dose of glucose was 50 g. given in 100 cc. water.

Time hrs.	Blood-sugar g. %	R.Q.	O_2 cc. per min.	CO_2 cc. per min.
		Subject *M.* Eunuchoid.		
0	0·072	0·88	259	228
½	0·100	1·01	240	242
1	0·104	1·02	212	217
1½	0·113	0·99	204	202
2	0·081	0·95	249	236
		Subject *C.* Cretin.		
0	0·087	0·90	86	78
½	0·085	1·00	82	82
1	0·133	1·07	78	80
1½	0·125	1·16	79	91
2	0·099	1·16	77	89

In such cases it must be supposed that the excess carbon dioxide is produced from the change of carbohydrate to fatty acid. In the case of dihydroxyacetone, however, we must at present regard the very high R.Q. associated with a large rise in oxygen consumption as pointing to powerful oxidation going on simultaneously with the formation of fat. The rapidity with which the oxygen absorption takes place points to direct oxidation in spite of the mass of evidence against it. The findings of Lambie and Redhead [1927] indicate that there occurs a rapid rise in the blood-dihydroxyacetone and, in the normal subject, a fall to low values in about an hour, corresponding approximately to the rise and fall in the oxygen used. It seems to us difficult to escape from the view that direct oxidation can go on at least to some extent, whilst simultaneously glycogen is being formed.

We must now consider the effect of dihydroxyacetone on the ketosis in Exp. 6 as compared with the effect of glucose in Exp. 4. In Exp. 4 the ketosis disappeared in about 1 hour 10 minutes after the sugar was taken and only the faintest traces of acetoacetic acid could be found during the succeeding 3¼ hours, when the excretion of ketonic acids began definitely to increase. This is a finding which has been made on many occasions.

With the dihydroxyacetone the results were quite different. First of all, there was no period throughout the whole experiment in which acetoacetic acid and β-hydroxybutyric acid could not be easily estimated. There was, indeed, a marked fall, which began about 1½ hours after the triose was taken and lasted for ½ hour, when the excretion per unit time began to increase again. This result is so definitely different from anything we have ever obtained under similar conditions with glucose, that we are forced to the conclusion that dihydroxyacetone in these conditions forms glycogen much less readily than glucose or that it is forming fat at the same time.

Again comparing Exps. 4 and 6 we note that in the case of glucose the disappearance of the ketones is simultaneous with the increase in oxygen intake, whilst in the case of dihydroxyacetone the rise in oxygen took place about an hour before the marked fall in ketosis. As there appears no reason to doubt that effective oxidation of glucose leads to antiketogenesis or ketolysis very rapidly, we suggest that the initial rapid rise in oxygen absorption in Exp. 6 was not for the purpose of oxidation of glucose. Later on, as glycogen was formed, antiketogenesis became obvious, although at no time was there

sufficient to establish complete keto-antiketogenic equilibrium; which may in part be due to the formation of fat. The fact that during the half-hour or so after the dihydroxyacetone was taken there occurred a marked rise in the ketone substances would make this latter assumption not improbable, since, as we saw in Exp. 1, this is what occurs when fat is fed in similar conditions.

Our results, then, suggest that dihydroxyacetone forms glycogen, at least in the starving organism, much less readily than glucose does and is less effective as an antiketogenic substance. Further, our findings do not lend themselves to the conception that this triose is an intermediate stage in the breakdown of the glucose molecule.

Preferential oxidation is always very difficult to demonstrate directly and interpretations of experiments on the respiratory metabolism are beset with difficulties. However obvious it may seem that direct oxidation is taking place, it is nearly always possible to give alternative explanations of any changes in R.Q. or oxygen absorption which may occur. In reporting the following three experiments on a case of extreme non-diabetic glycosuria of the obese type, we are sensible of the danger in interpreting the data on lines of preferential oxidation, but we suggest that the results are at least suggestive of it. When dealing with the sugars, most of which give rise to glycogen, which in its turn can only give rise to glucose on hydrolysis, the idea is naturally developed that glycogen is a necessary intermediate step before any of them can be metabolised. It seems probable that ultimately all the sugars are utilised by way of glycogen, but it is difficult to avoid the conclusion that some of them may be oxidised directly.

In the following experiments the conditions were similar to those usually employed in estimating the effects of sugars on the respiratory metabolism, viz. 15 hours without food, 1 hour complete rest, estimation of the fasting metabolism and then administration of the sugar.

Subject *V*. Age 50. Weight 15 st. 10 lbs. Obese glycosuric. No ketosis at any time.

Time mins.	Blood-sugar g. %	R.Q.	O_2 cc. per min.	CO_2 cc. per min.	Urinary sugar as g. glucose
		50 g. glucose.			
0	0·336	0·84	258	216	
30	0·327	0·82	255	209	
60	0·396	0·79	255	203	24·46
93	0·400	0·75	275	205	in 150 mins.
125	0·376	0·75	270	203	
150	0·312	—	—	—	
		50 g. laevulose.			
0	0·268	0·87	297	259	
40	0·286	0·89	316	281	
68	0·291	0·95	285	271	19·92
106	0·304	0·94	297	286	in 270 mins.
139	0·286	0·81	292	237	
178	0·271	0·83	291	240	
		50 g. galactose.			
0	0·289	0·75	283	213	
35	0·336	0·78	307	239	19·08
65	0·375	0·98	276	271	in 260 mins.
100	0·341	0·91	269	245	
135	0·310	0·77	273	210	

In such a case as this the suggestion made by Cathcart and Markowitz [1927, 2], that the higher R.Q.s obtained after laevulose and galactose may bear some relationship to the rapid removal of sugar from the circulation, does not apply, for here we have an extreme deficiency in the power to store carbohydrate in the liver. So much is this the case that this type of patient shows a remarkable resistance to insulin even in the fasting condition. This being so, it seems to us likely that the higher R.Q.s obtained after laevulose and galactose are true indices of at least some preferential oxidation. There seems no reason to suppose that these sugars have led to a diminished gluco-neogenesis to a greater extent than glucose and even if this be insisted upon it would still be something in the nature of a preferential metabolism.

In view of the fact that dihydroxyacetone brings about a marked hypoglycaemia in man, it was of interest to see if a similar effect could be demonstrated in the rabbit. The following results were obtained in two rabbits which have been frequently used for experiments with glucose, and the blood-sugar curves of which have often been determined and found of the same nature. The figures were obtained in each case after 24 hours without food.

Rabbit 1. 2·5 kg.

4 g. glucose in 20 cc. water, *per os*		4 g. dihydroxyacetone in 20 cc. water *per os*	
Time mins.	Blood-sugar g. %	Time mins.	Blood-sugar g. %
0	0·109	0	0·111
30	0·162	20	0·129
60	0·137	35	0·115
90	0·106	65	0·175
120	0·109	95	0·156
		125	0·135
		155	0·146
		185	0·133
		215	0·129
		245	0·117

Rabbit 2. 2·7 kg.

0	0·104	0	0·117
30	0·166	30	0·122
60	0·129	60	0·143
90	0·113	90	0·135
120	0·113	120	0·131
		150	0·110
		180	0·131

It would thus appear that in the rabbit there does not occur the same hypoglycaemic sequence of blood-sugar values as occurs in man. Further experiments are necessary before any interpretation can be offered.

Summary.

1. The metabolic effects of glucose and dihydroxyacetone in the normal and starving human subject are compared. The much greater oxygen intake and higher R.Q.s following the administration of dihydroxyacetone as compared with glucose, found by other observers, are confirmed.

2. Dihydroxyacetone is not an effective antiketogenic substance. Although it produces a definite diminution in the ketosis of starvation, its power in this direction is much less than that of glucose. It is pointed out that such antiketogenesis as it does exert does not occur simultaneously with the maximum rise in oxygen intake, which is the case with glucose.

3. The question of preferential oxidation is discussed.

4. The mechanism of the production of carbohydrate intolerance in starvation is discussed.

5. The hypoglycaemia which occurs in man after administration of dihydroxyacetone does not occur in the rabbit.

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LXII. THE DEAMINATION AND SYNTHESIS OF l-ASPARTIC ACID IN THE PRESENCE OF BACTERIA.

By ROBERT PERCIVAL COOK
and BARNET WOOLF (*Beit Memorial Research Fellow*).

From the Biochemical Laboratory, Cambridge.

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It was shown by Quastel and Woolf [1926] that when *l*-aspartic acid is incubated anaerobically with resting *B. coli communis* at 37° and p_H 7·4, complete deamination occurs, fumaric acid appearing in the early stages of the reaction, to be replaced later by succinic acid. If fumaric acid and ammonium chloride are incubated under the same conditions, there is first an ammonia uptake and a synthesis of *l*-aspartic acid, followed by the reappearance of all the ammonia, at which point nothing but succinic acid can be found in the medium. In the presence, however, of certain inhibiting substances, such as 2 or 4 % propyl alcohol, *M*/20 or *M*/10 sodium nitrite, or toluene, the production of succinic acid is completely inhibited. Under these circumstances, only fumaric acid is formed from aspartic acid, and aspartic acid from fumaric acid and ammonia, the reaction being a true chemical equilibrium for which equilibrium curves were obtained and the constant calculated. Resting *B. coli* thus appears to be able to effect two distinct chemical reactions, one reversible

$$COOH . CH_2 . CH(NH_2) . COOH \rightleftharpoons COOH . CH : CH . COOH + NH_3$$

which persists in the presence of inhibitors which completely abolish the other, irreversible reaction

$$COOH . CH_2 . CH(NH_2) . COOH + 2H \rightarrow COOH . CH_2 . CH_2 . COOH + NH_3.$$

The object of the work to be described was to determine how far the power to effect these two reactions is distributed among other species of bacteria.

The following organisms were used:

Strict aerobes	Facultative anaerobes	Strict anaerobes
B. alkaligenes	*B. pyocyaneus*	*B. sporogenes*
B. subtilis	*B. prodigiosus*	*B. histolyticus*
B. phlei (Timothy Grass Bacillus)	*B. proteus*	*B. tertius*
B. megatherium	*B. fluorescens*	

The organisms were grown in Cole and Onslow's tryptic broth. The aerobes and facultative anaerobes were grown aerobically in Roux bottles containing 150 cc. of broth, which were inoculated from 12-hour old broth cultures. The

time of growth was usually 2 or 3 days, except in the case of *B. megatherium* and *B. phlei*, which were allowed to grow for 10 days. The strict anaerobes were grown in large flasks, either anaerobically, or aerobically in presence of 0·01 % cysteine [Quastel and Stephenson, 1926] for about a fortnight.

After growth, the organisms were centrifuged from the broth, and washed three times by centrifuging with 0·85 % saline. They were then emulsified in saline, the emulsion being put in the shaking machine for an hour or two; in this way it was possible to obtain a homogeneous emulsion, even with granular growths like *B. subtilis* and *B. phlei*, and slimy growths like *B. prodigiosus*. The emulsion was finally aerated for 4 hours, and was then ready for use. It was stored in the refrigerator. Although it was generally used fresh, there was no marked change in its activity for at least 2 months. The various emulsions were made very roughly comparable by diluting with saline until 1 cc., when diluted to 50 cc., showed a standard opacity. The dilution chosen was such that the growth from one Roux bottle of a prolific organism like *B. alkaligenes* generally corresponded to about 10 cc. of the emulsion. At least two strains of each organism, obtained from different sources, were used, and always gave concordant results.

All the experiments were done anaerobically at p_H 7·4 and 37°. Reaction mixtures, containing known concentrations of sodium aspartate, sodium fumarate, and ammonium chloride, with the organism to be tested, and the inhibitor (if any) were made up, and placed in filtering flasks. All the reaction mixtures contained at least half their volume of Clark and Lubs's phosphate buffer, p_H 7·4. The course of the reaction was followed by frequent estimations of the free ammonia, by the modified Van Slyke aeration method described by Quastel and Woolf [1926]. A sample was taken for an initial estimation, and the filtering flask was stoppered, exhausted at the water-pump, and incubated, and the flask was re-evacuated and incubated after each subsequent sample. Duplicate flasks were put up, which were used for the isolation of aspartic, fumaric or succinic acid, as described later, when the curve of ammonia uptake or output indicated that a suitable stage had been reached.

The organisms were found to fall into two distinct groups, one containing all the facultative anaerobes and the other all the strict aerobes and strict anaerobes.

Results with facultative anaerobes.

All the facultative anaerobes were found to behave in a similar manner to *B. coli communis*, giving both the equilibrium and the reduction in the absence of inhibitors, but only the equilibrium in the presence of inhibitors. The equilibrium point was the same with the various organisms and inhibitors, and the velocity was similar for equal concentrations of the various emulsions.

The concentrations of reactants generally employed were $M/10$ *l*-aspartic acid, and $M/10$ fumaric acid + $M/10$ ammonium chloride. The inhibitors used were 2 % and 4 % propyl alcohol, $M/10$ sodium nitrite, and toluene. The

bacterial emulsion was generally used at a strength of 1 cc. in 50 cc. of the reaction mixture, and the reaction was complete in from 3 to 5 days.

Fig. 1 shows a typical set of reaction curves, obtained with *B. fluorescens*, both without inhibitor (curves *A* and *F*) and in presence of 4 % propyl alcohol (curves *AP* and *FP*). The reaction mixtures were made up as follows.

	M/2 sodium aspartate cc.	*M*/2 sodium fumarate cc.	*M* NH_4Cl cc.	C_3H_7OH cc.	Buffer p_H 7·4 cc.	Bacterial emulsion cc.
A	10	—	—	—	39	1
F	—	10	5	—	34	1
AP	10	—	—	2	37	1
FP	—	10	5	2	32	1

The curves exhibit the following features.

Curve A. A rapid initial ammonia output which later slows down. During this period fumaric acid is being formed. There is then a rapid linear output of ammonia to completion, after which only succinic acid can be isolated.

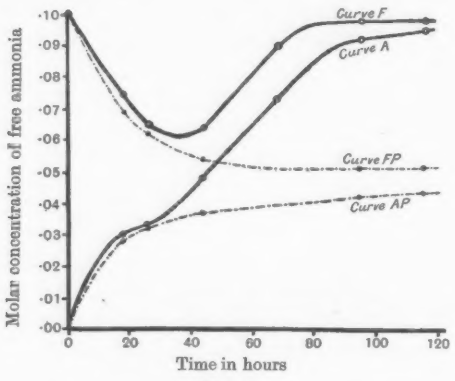

Fig. 1.

Curve F. There is an initial ammonia uptake by fumaric acid, with the production of aspartic acid. This is followed by a linear output of ammonia, parallel to that given by curve *A*, at the end of which only succinic acid is present.

Curve AP. An ammonia output, during which fumaric acid is produced, no succinic acid being detectable.

Curve FP. A synthesis of aspartic acid until the equilibrium concentration is reached.

Curves *A* and *F* differ from the corresponding curves obtained with *B. coli* by Quastel and Woolf in one respect. With *B. coli*, curve *F* invariably crosses curve *A* before the linear portion is reached. With the other facultative anaerobes this crossing is the exception. We have observed it in two cases only, once with *B. fluorescens* and once with *B. pyocyaneus*. Curves *AP* and *FP*, though close approximations to true equilibrium curves, are subject to

a small disturbing factor, which prevents the curves from meeting. They approach closely together, and then rise very slowly parallel to one another. The equilibrium value can be taken, to a first approximation, as being half-way between the curves at the point where the rise begins. The more closely the curves approach, *i.e.* the less prominent the disturbing factor, the lower the concentration of ammonia found at the equilibrium point. Some typical values of this equilibrium concentration are shown in Table I, for different organisms and inhibitors. Values already published for *B. coli* are given for comparison. The figures represent molar concentration of free ammonia at equilibrium, starting with reaction mixtures containing $M/10$ aspartate and $M/10$ fumarate + $M/10$ ammonia, and it will be seen that the equilibrium point is the same in all cases, allowing for the error due to the disturbing effect already mentioned.

Table I.

	Inhibitor			
Organism	2 % C_3H_7OH	4 % C_3H_7OH	Toluene	$M/10$ sodium nitrite
B. fluorescens	0·046	0·046	0·049	0·050
B. prodigiosus	0·049	0·049	0·048	0·051
B. proteus	0·045	0·047	0·047	0·047
B. pyocyaneus	0·048	0·049	0·048	0·049
B. coli	0·047	0·047	0·047	0·046

All controls, such as aspartic acid or fumaric acid and ammonia without organism, fumaric acid and organism without ammonia, ammonia and organism without fumaric acid, with and without the inhibitors used, were negative. Well-boiled emulsions of the organisms were unable to effect either the equilibrium or the reduction to succinic acid.

Results with strict aerobes and strict anaerobes.

All the strict aerobes and strict anaerobes tested show an entirely different type of behaviour. They give no sign of an equilibrium, but seem able to effect the reduction of aspartic acid to succinic acid as readily as the facultative anaerobes. When aspartic acid is incubated with one of these organisms in the presence of an inhibitor there is no ammonia output, even when 20 cc. of bacterial emulsion is used in 50 cc. of reaction mixture, and the experiment is extended for 2 weeks. Similarly, there is no ammonia uptake by fumaric acid, although the concentration of the organisms may be raised to half the volume of the reaction medium, and the fumaric acid be present in a concentration up to $M/2$, with ammonium chloride at molar concentration, over a period of a fortnight. Controls with a facultative anaerobe at these concentrations give an ammonia uptake to equilibrium in an hour or two.

In the absence of inhibitors, there is usually no ammonia uptake by fumaric acid. Aspartic acid, however, is completely deaminated, the only product being succinic acid. No fumaric acid is detectable at any stage of the reaction.

The course of the ammonia output from aspartic acid in two typical cases is shown in Fig. 2, one curve being obtained with an aerobe and the other with

an anaerobe. The aspartic acid was $M/10$, and the concentration of bacterial emulsion was 1 cc. in 50 cc. of the medium. It will be seen that the curves consist of two portions, a period of very slow ammonia output followed by a rapid linear deamination to completion. The relative durations of these two portions seem to be different for each emulsion, different preparations of the same organism giving sometimes a short latent period and a slow linear period and sometimes the reverse. There is no correlation between the relative durations and the bacterial species.

Although there is never any ammonia uptake by fumaric acid when inhibitors are present, there is sometimes a disappearance of ammonia in their absence. This phenomenon is especially noticed with *B. alkaligenes*. When this occurs, there is also a slow ammonia uptake with aspartic acid after deamination has proceeded to completion. This makes it very unlikely that the uptake is due to a synthesis of aspartic acid. It is reported by Quastel and Wooldridge [1925] that well-washed *B. alkaligenes* still shows great reducing activity in the Thunberg tube towards methylene blue, indicating that there are reactive

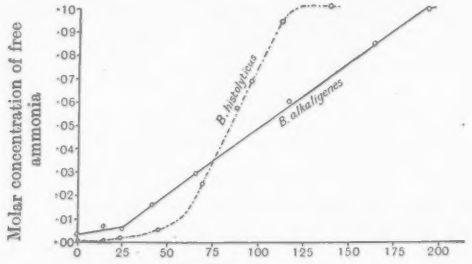

Fig. 2.

substances present which cannot be washed away. It seemed possible that these might be the cause of the disappearance of ammonia. An attempt was made to test this by washing a preparation of *B. alkaligenes* very thoroughly, in the hope that both reducing activity and ammonia uptake would diminish. It proved impossible, however, to increase the reduction time by repeated washing. 1 cc. of the original emulsion was placed in a Thunberg tube with 3 cc. of buffer and 1 cc. of 1/5000 methylene blue, and the reduction time at 37° was 9 minutes. A portion of the emulsion was washed four times by centrifuging with saline, made up to the original volume, and aerated for 6 hours. The reduction time was still 9 minutes. That the ammonia uptake was not due to a synthesis of aspartic acid was shown, however, by putting up a reaction mixture containing $M/10$ fumaric acid + $M/10$ ammonium chloride, with half its volume of this emulsion. In 5 days 15 % of the ammonia had disappeared, a much higher figure than usual. If aspartic acid had been produced in this quantity, it would have been easy to isolate. On attempting an isolation none was obtained, and the copper filtrate was pale blue, instead of

the dark blue obtained with even a trace of aspartic acid. It seems clear, therefore, that the uptake is due to some disturbing reaction that, unlike the aspartic-fumaric equilibrium, is stopped by inhibitors. With ammonium chloride alone, this emulsion showed no ammonia uptake.

Isolation of aspartic acid.

The reaction mixture was heated to boiling and saturated with solid copper sulphate. Boiling was continued for half an hour and the light blue precipitate was filtered off. The clear, deep blue filtrate was allowed to stand in the refrigerator for 3 days, when crystallisation of the copper aspartate was complete. The crystals were filtered off, well washed with cold water, and twice recrystallised from hot water. They were then dried to constant weight over sulphuric acid in the desiccator. Free aspartic acid was obtained by treating the copper salt with H_2S and decolorising if necessary with a little animal charcoal.

For identification, copper and nitrogen determinations were made on the copper salt, and melting-point and nitrogen determinations on the free acid. The rotation was also measured in presence of 3 mols. of HCl.

Copper salt. Found: Cu, 25·5–25·9 %; N, 5·37–5·49 %. Calculated for $C_4H_5O_4NCu$, $3H_2O$ Cu, 25·6 %; N, 5·63 %.

Free acid, M.P. in sealed tube, 269°–271° (uncorr.). Admixture with authentic aspartic acid did not alter the M.P., or raised it by not more than one degree.

N, found, 10·1–10·3 %. Calculated, 10·5 %.

Average for $[\alpha]_{Hg\ green}$ +27°.

The yield of copper aspartate was between 60 and 80 % of that calculated from the reaction curve.

Isolation of succinic acid.

The organisms were removed by centrifuging and filtering through kieselguhr, 10 % phosphoric acid was added and the mixture thoroughly extracted with ether. The ether extract was evaporated to dryness, and the product taken up in water, decolorised with animal charcoal, and again evaporated to dryness. The crystalline residue of succinic acid had no decolorising effect on alkaline permanganate, and the different specimens melted between 184° and 186° (uncorr.). Admixture with authentic succinic acid either did not affect the melting-point or raised it by half a degree. The yield was from 40 to 60 % of the theoretical.

Isolation of fumaric acid.

The reaction mixture was freed from bacteria by centrifuging and filtering through kieselguhr, and toluene or propyl alcohol, if present, was removed by evaporation. 10 % of phosphoric acid was then added and the mixture thoroughly extracted with ether. The ether extract was evaporated to dryness and the residue was dissolved in water and precipitated with a strong solution of silver nitrate. The white precipitate of silver fumarate was filtered off,

washed, and decomposed with H_2S. The filtrate from the Ag_2S was decolorised with charcoal and evaporated to dryness. The residue of fumaric acid decolorised alkaline permanganate solution in the cold. It did not melt in an open capillary tube, but showed signs of sublimation just above 200°. In a closed capillary tube it melted between 278° and 279·5° (uncorr.), admixture with authentic fumaric acid having no effect on the melting-point. The yield was between 30 and 40 % of the theoretical.

Experiments with other amino-acids.

Preliminary experiments with glycine, alanine and glutamic acid indicate that all these substances are deaminated by all the organisms used when inhibitors are absent, but that no ammonia is given out when inhibitors are present.

Discussion.

It seems clear from the results described above that there are present in bacteria two distinct mechanisms capable of dealing with aspartic acid.

1. A mechanism which effects an irreversible reductive deamination to succinic acid, its time course being represented by a latent period followed by a straight line to completion. This mechanism is present in all the bacteria tested and is completely stopped by inhibitors.

2. A mechanism which effects a reversible equilibrium between aspartic acid and fumaric acid and ammonia. This mechanism is present only in facultative anaerobes, and is unaffected by inhibitors.

It is possible, therefore, by varying the conditions, to obtain curves showing the effect of mechanism 1 only (Fig. 2) or mechanism 2 only (curves *AP* and *FP*, Fig. 1) or a summation effect of the two mechanisms (curves *A* and *F*, Fig. 1).

Since the second mechanism acts in the presence of substances known to inhibit the growth of the bacteria concerned, there can be no question that it is due to the true "resting" or non-proliferating organism. Experiments with methylene blue in the Thunberg tube show that facultative anaerobes like *B. coli*, *B. proteus* and *B. prodigiosus* possess far more general activating powers than strict aerobes like *B. alkaligenes* [Quastel and Wooldridge, 1925] and strict anaerobes (unpublished results by these authors). It seems possible that the fumaric-aspartic mechanism is bound up with the activating mechanisms of the organism, and experiments are in progress to test this point.

Since the first mechanism is stopped by growth inhibitors, there is a possibility that it may be due to the growth of the organisms in the reaction mixture. This was the suggested explanation of Quastel and Woolf [1926]; they postulated that anaerobic growth might occur on the protein of dead bacteria in the emulsion, the aspartic acid acting as hydrogen acceptor. This explanation now seems unlikely for several reasons. Firstly, one would hardly expect strict aerobes to be able to grow anaerobically in this way. Secondly, slow-growing organisms like *B. megatherium* seem able to bring about the reaction as rapidly

as the more prolific organisms. Thirdly, the amount of bacterial substance present can hardly account for the complete reduction observed.

The only possible alternative explanation seems to be that the aspartic acid undergoes an oxidation-reduction process, a part being oxidised to provide for the reduction of the rest. The yields of succinic acid obtained leave an ample margin for this to be possible. If this explanation be correct, the peculiar nature of the curve might be explained as follows. During the latent period a very slow oxidation is going on, resulting in the appearance of some reactive product (*e.g.* pyruvic acid). When this substance has reached a certain concentration, it is oxidised, reducing several molecules of aspartic acid in the process. The straight line portion is then reached, being an expression of the steady production of the hypothetical reactive substance. Experimental tests of this supposition are in progress.

The teleological significance of this reaction may be that the organism needs a mechanism to disengage ammonia from amino-acids in order that it may enter into the synthetic processes of growth. The fact that other amino-acids are deaminated tends to support this view, which, if true, would fit in with the theory that when a bacillus grows on a medium containing amino-acids as the source of nitrogen, the nitrogen must be turned into ammonia before it can be utilised.

Summary.

Eleven species of bacteria have been tested. At 37° and p_H 7·4 they all deaminate aspartic acid anaerobically to succinic acid. This reaction is completely prevented by the presence of inhibitors, such as 2 or 4 % propyl alcohol, $M/10$ sodium nitrite, or toluene. In addition, all the four facultative anaerobes can effect a reversible equilibrium

$$\text{aspartic acid} \rightleftharpoons \text{fumaric acid} + \text{ammonia},$$

giving the same equilibrium constant as that already observed with *B. coli communis*. The four strict aerobes and three strict anaerobes used show no trace of this reaction, which is unaffected by the presence of the inhibitors. Well-boiled bacteria have no deaminating effect on aspartic acid.

Preliminary work indicates that all the bacteria used will completely deaminate glycine, alanine and glutamic acid in the absence of inhibitors. When inhibitors are added there is no ammonia output.

It is a pleasure to express our appreciation of the interest taken by Sir F. G. Hopkins in this work. We wish to thank Mr E. G. D. Murray, of the Pathological Department, Cambridge, for kindly supplying us with some of the bacterial cultures used.

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LXIII. THE SEXUAL GLANDS AND METABOLISM.

V. THE INFLUENCE OF LIPOID EXTRACTS OF THE TESTES AND PROSTATE ON THE NITROGEN METABOLISM OF RABBITS AND ON THE DEVELOPMENT OF THE GENITAL ORGANS OF RATS.

By VLADIMIR KORENCHEVSKY.

From the Lister Institute, London.

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Historical.

Testes.

Iscovesco was the first investigator to study the lipoids of the gonads [1912, 1, 2]. He [1907–1914] extracted the lipoids of several tissues and glands, including the testis, and came to the conclusion that all the organs which he had investigated contained at least one specific lipoid which, when introduced into the organism, caused an increase in the function of the organ from which the lipoid had been isolated. Iscovesco therefore called these lipoids *homostimulines*. From a study of the action of various fractions of the lipoids he concluded that some of them are physiologically indifferent, others antagonistic to homostimulines. These he called *heterostimulines*.

Briefly, Iscovesco's method of isolation of the lipoids is as follows. To the minced tissue about three volumes of 95 % alcohol are added. After a few hours the alcoholic extract is filtered off and from this group I (alcohol-soluble) lipoids are isolated. The remaining tissue is desiccated, powdered and successively extracted with ether (group II, ether-soluble lipoids), with acetone (group III, acetone-soluble lipoids), with chloroform (group IV, chloroform-soluble lipoids) and again with alcohol (group V, alcohol-soluble lipoids). Each of these groups is further divided, as, for example, group II. The extract left after evaporation of the ether is mixed with light petroleum, by means of which it is divided into an insoluble portion, *A*, and a soluble portion, *B*. The *B* portion is evaporated and again dissolved in ethyl ether. If there is any precipitate, it is removed by centrifuging. The clear liquid is mixed with two volumes of acetone and left in the cold room for 24 hours. A precipitate, *C*, results, which is gathered on a filter-paper. The filtrate is concentrated and again left in the cold room and a second precipitate, *D*, is collected by filtration. The filtrate is evaporated and the residue extracted with boiling alcohol in

which it is completely soluble. After cooling for 24 hours it is separated into a precipitate, *E*, and the material in solution, *F*. Nearly all the cholesterol is collected in *E*. In their turn, each of these *A–F* fractions is treated in succession with light petroleum, ethyl ether, cold and boiling alcohol. In this way fractions *a*, *b*, *c* etc. are made from each of the previous fractions *A*, *B*, *C*, *D*, *E* and *F*.

Testicular specific homostimulines were found in the second ether-soluble group, fraction II *Fb* and in the fifth alcohol-soluble group, fraction V *Dc*. Iscovesco emphasises the fact that the testes contain several non-specific heterostimuline lipoids, some of which have a strong antagonistic influence and some of which are physiologically inactive.

In 2 or 3 months 34 to 45 subcutaneous injections of 0·01 g. per kg. of fractions II *Fb* or V *Dc*, dissolved in olive oil, produced hypertrophy of the testes in rabbits, the testes becoming two or three times heavier than those of the uninjected control animals. The general growth and condition of the rabbit also improved. The total amount injected was from 0·5 to 0·6 g. of the lipoid fraction. Doses, which were too large (about 0·03 g.), of fraction II *E* produced in some animals paralysis of the hind legs. It is to be regretted that the number of experiments performed and some important details were omitted from this paper.

Hoskins [1927], in collaboration with Brown and Lee, was unable to obtain any influence on the voluntary activity of male rats with lipoid extracts prepared by Allen and Doisy's method. Feller [1913, 1920] found among the lipoids extracted with alcohol and light petroleum from placenta, foetal membranes, corpus luteum, ovary and testicles, a fraction which produced hypertrophy of the uterus. The lipoid extracted from testes produced atrophy of the spermatogenic cells of the testes, but a slight hypertrophy of the interstitial cells, with an increase of their lipoid granules. No change was observed in the prostate or seminal vesicles. The corresponding lipoid fractions from the ovary and from the placenta produced the same change in testicular cells. This interesting fact was corroborated by Herrmann and Stein [1916]. They injected a lipoid fraction of placenta, or of corpus luteum, which they had prepared, into male rabbits. Atrophy of the testes, prostate and seminal vesicles resulted. Histologically the spermatogenic cells of the gonads were found to be atrophied, while the interstitial tissue seemed to have increased slightly. In another experiment they removed one testicle from a rabbit. The remaining testicle, after the lipoid injections, was found, on comparing it with the testicle which had been removed, to have developed the same changes as those mentioned above.

Glimm and Wadeln [1926], using a lipoid fraction extracted from placenta, and Laqueur, Hart and De Youngh [1925, 1926, 1927], by injecting their water-soluble oestrus hormone (menoform), obtained from follicular fluid, inhibited the growth of the genital organs of the male animals.

Schroeder [1920] produced hypertrophy of the *utriculus masculinus* in the male rabbit by injecting a lipoid extract which he had prepared from placenta.

Prostate.

We do not know of any investigations in which lipoid extracts of the prostate have been shown to have any physiological action.

Experimental.

In order to discover whether the various fractions isolated by us contained specific sexual hormones, two kinds of tests have been applied: first, the influence of isolated fractions on the growth of the genital organs of normal and castrated animals, and second, the influence on the nitrogen metabolism which is partly controlled, as our previous investigations have shown, by the inner secretion of the sexual glands.

Technique.

For the preparation of lipoid extracts bulls' testes or prostate were used, and were brought from the slaughterhouse just after the animals had been killed. After the removal of fat and membranes, the glands were minced in a special Latapie's grinding machine. The method of fractionation was based on that of Doisy *et al.* [1924], successfully applied by them to the ovary. This method and various modifications of it were used in the attempt to isolate a testicular hormone. In the following account the former will be called the American method. In the American method the extracts are heated. As the degree of thermostability of testicular lipoids is not known, it seemed reasonable to modify it so as to avoid the use of high temperatures. This method will be called the ether method.

In the American method, after the first two alcoholic extractions of the tissue, the residue is thrown away. Since preliminary experiments showed that this residue still contained lipoids, it was decided to extract these. It was also considered advisable to eliminate from the fractions the insulin-like substances which are present in large amount in several tissues and particularly in testes. As has been shown previously [Korenchevsky and Carr, 1925, 1], these substances mask the effect given by the sexual hormones. To remove them, the final processes were carried out with anhydrous alcohol, ether and acetone, in which insulin is insoluble.

The practical details of the following method, which is described below, were carried out with the kind assistance of Mr F. H. Carr, to whom and to Messrs British Drug Houses, Ltd., my thanks are due. To test the technique of the method of preparation of sexual lipoids, Mr Carr was so kind as to have prepared in the Laboratory of Messrs British Drug Houses, Ltd., one batch of the extracts. The chemical and physiological properties were investigated at the Lister Institute and were found to be identical with those of the batches, prepared by us at the Lister Institute.

The method, as used, was the following. To the minced tissue of the glands an equal volume of 96 % alcohol was added, and after standing for 1 to 2 hours at room temperature the mixture was left in the cold room overnight. The next

day the mixture was filtered on a Büchner funnel and the residue pressed in a hydraulic press. The fluid so pressed out was added to the filtrate. The residue was re-ground in a mortar with an approximately equal volume of absolute alcohol, filtered, and the filtrate added to those already obtained. The mixture of all the filtrates above was called *A*.

The residue from the filtrate *A* was ground once more with absolute alcohol and filtered. The same procedure was repeated twice more and the filtrates combined. The residue was then extracted in a Soxhlet apparatus with ether and afterwards with boiling acetone. After evaporating off the ether and acetone in a vacuum the second alcoholic filtrates were added to the residue giving the second mixture *B*.

The mixture *A* was distilled to dryness in a vacuum and the residue divided into two equal parts, one part being used for the ether method (lipoid fraction No. 1) and the other for the American method (lipoid fraction No. 2). The mixture *B* gave in the same way lipoid fractions No. 3 (ether method) and No. 4 (American method).

In the following account all the testicular extracts are denoted by the letter *T* and the prostate extracts by the letter *P*. For example, P_3 denotes the lipoid fraction No. 3 prepared from prostate. The American method was used as it was described by Doisy *et al.* [1924]. The ether method was carried out as follows. The dry residue from the mixture *A* or *B* was washed into large centrifuge tubes with about 100 cc. of anhydrous ether, in which it is almost completely soluble. It was then left for a few hours or until the next morning in the cold room. The mixture was centrifuged while still cold and the clear ether extract poured off, the precipitate being thrown away. The fluid was distilled to dryness in a vacuum and the residue so obtained kept in nitrogen in the dark in the cold store. The lipoid fractions No. 2 and No. 4 were kept in the same way in order to prevent oxidation and decomposition by light and air. The injections were prepared by emulsifying, in a mortar, the weighed amount of the lipoid extract with saline solution, made slightly alkaline with $NaHCO_3$. Good emulsions were obtained in this way.

The nitrogen metabolism experiments were performed by the method described elsewhere [Korenchevsky and Carr, 1925, 1, 2].

The influence of injections of lipoid extracts on the growth of the genital organs and thymus of normal and castrated rats.

One hundred and two rats (belonging to 16 litters), of which 92 were castrated and 10 normal, were used in the experiments. Each litter was divided into a control group and an injection group, the injections being made a few days after the operation had been performed. Altogether 17 injections of from 0·02 g. to 0·05 g. of the lipoid extracts were made during 45 days. The total number of control rats was 34, the number injected with T_1 6, with T_2 8, with T_3 8, with T_4 5, with P_1 3, with P_2 6, with P_3 8, with P_4 3, with a mixture of $T_1 + P_1$ 3, with $T_2 + P_2$ 3, with $T_3 + P_3$ 11, and with $T_4 + P_4$ 4. At the end

of the experiment the rats were killed and the weights of the prostate, the seminal vesicles and the thymus and the measurements, in mm., of the penis and the seminal vesicles of the injected animals compared with those of the control animals. It is well known that the penis, prostate and seminal vesicles atrophy after castration, whilst the thymus persists for an unusually long time or may even hypertrophy. No difference was observed between the injected and the control rats in either normal or castrated litters.

The influence of lipoid extracts on the nitrogen metabolism of rabbits.

Testicular extracts. Twenty-eight experiments were performed on 11 rabbits of which 6 were normal, 2 castrated and 3 thyroidectomised. The amount of each extract injected each day, per kg. of rabbit weight, varied from 0·03 to 0·2 g. When a mixture of testicular and prostatic extracts was injected simultaneously the amounts of each of the two extracts were equal to half of that which was injected singly, for the purpose of comparing the results. In the control periods saline solution was injected. In the tables the total amount, per kg. of rabbit weight, injected is shown. In most cases the experiment was divided into four periods: a first control period, of 2 to 3 days before the injections, the period of injection of from 2 to 3 days, followed by two or three control periods, each of from 2 to 3 days. Usually at least 1 month divided two experiments on any one rabbit, but in some experiments, when the nitrogen metabolism had returned to normal, a second injection was made about a week after the first. In order that the number of tables may be decreased we give one in detail, as an example, and four tables giving the general results showing the changes in the urinary flow, in the excretion of the nitrogen of the urine and in the nitrogen balance.

Table I. *The influence of simultaneous injection of the extracts of testes T_3 and of prostate P_3 upon the nitrogen metabolism and urinary flow of the normal rabbit No.* 7.

No. of period	Length of period hrs.	Weight of rabbit g.	Total nitrogen intake g.	Volume of urine excreted cc.	Nitrogen in urine g.	Nitrogen in faeces g.	Total nitrogen output g.	Balance of nitrogen g.	Remarks
I	48	2910	10·42	354	6·65	2·86	9·51	+0·91	Injected saline
II	48	2860	10·42	616	8·30	2·86	11·16	−0·74	Inj. 0·5 g. T_3+P_3 (0·25 g. of each)
III	48	2850	10·42	326	6·87	2·86	9·73	+0·69	Injected saline
IV	72	2830	10·42	232	6·50	2·86	9·36	+1·06	,,
V	48	2840	10·42	238	6·35	2·86	9·21	+1·21	,,

Subcutaneous injections of the testicular lipoid extracts T_1, T_2, T_3 and T_4 (Table II) produced only small changes of varying character in the excretion of urinary nitrogen and in the balance of nitrogen. For example, the greatest increase of excreted nitrogen in the urine was 5·7 % above normal, while the greatest decrease was 6·7 %. In the control period following the injection

period the change persisted, except in one experiment, in a less marked degree. The dose injected did not influence the results obtained.

Although in some experiments the change of urinary flow was considerable, at the same time this was inconsistent. For example, in two experiments the change was 43·3 % and 25·3 % above normal and in another two experiments 14·4 % and 16·3 % below normal (Table II).

Table II. *The influence of subcutaneous injections of lipoid testicular extracts Nos.* 1, 2, 3 *and* 4 *upon the nitrogen metabolism and urinary flow of rabbits.*

No. and description of rabbit	Total amount in g. per kg. and kind of extract injected		Percentage change in excretion of urine		Percentage change in nitrogen of urine		Change in nitrogen balance	
			Period of injection %	Following control period %	Period of injection %	Following control period %	Period of injection g.	Following control period g.
1. Castrated	0·27	T_1	−16·3	−24·0	−3·4	−7·1	+0·30	+0·63
1. Castrated	0·17	T_2	+ 1·3	+ 6·3	+1·9	+6·9	−0·14	−0·52
6. Castrated	0·18	T_2	+25·3	−25·7	−1·5	−3·9	+0·15	+0·38
28. Normal	0·21	T_3	− 1·6	−16·7	−0·1	−3·4	+1·04	+1·05
25. Normal	0·16	T_3	+43·3	+ 0·8	+5·7	+1·0	−0·47	−0·08
2. Thyroidect. and castrated	0·45	T_3	− 1·5	−19·5	−2·3	−5·3	+0·19	+0·43
8. Thyroidect. and castrated	0·21	T_4	−14·4	−29·9	−6·7	−8·6	+0·26	+0·43

Note. In Tables II to IV the figures marked + (increase) or − (decrease) denote the changes from the normal, *i.e.* the control period previous to the injections.

Table III. *The influence of subcutaneous injections of lipoid prostatic extracts Nos.* 2 *and* 3 *upon the nitrogen metabolism and urinary flow of rabbits.*

No. and description of rabbit	Total amount in g. per kg. and kind of extract injected		Percentage change in excretion of urine		Percentage change in nitrogen of urine		Change in nitrogen balance	
			Period of injection %	Following control period %	Period of injection %	Following control period %	Period of injection g.	Following control period g.
28. Normal	0·09	P_2	+ 1·9	− 4·0	+4·1	− 2·5	−0·40	+0·24
6. Castrated	0·14	P_2	− 2·2	− 9·3	+7·5	− 2·2	−0·68	+0·20
19. Normal	0·10	P_3	+ 7·2	−14·0	+7·9	+ 5·0	−0·73	−0·46
25. Normal	0·14	P_3	+ 1·4	−13·6	+0·9	− 9·7	−0·09	+0·96
19. Normal	0·20	P_3	+16·0	− 8·7	+8·2	+ 3·2	−0·59	−0·23
15. Normal	0·16	P_3	− 9·8	−12·1	+4·6	+10·7	−0·36	−0·98
6. Castrated	0·14	P_3	+ 1·3	+15·4	−2·1	− 4·9	+0·20	+0·48

Prostatic extracts. Of these, only two fractions, P_2 and P_3, were investigated as short preliminary experiments showed that the fractions P_1 and P_4 had, within the limits of physiological fluctuations, only a small and uncertain influence.

Of seven experiments (Table III) a slight increase of urinary nitrogen and a negative nitrogen balance was noticed in five experiments, while in two experiments there was practically no change. In the control periods following the period of injection the same change of the nitrogen metabolism was still

present or became reversed. The greatest increase of nitrogen excretion in urine was 8·2 % in the injection period and 10·7 % in the control period. There was no appreciable difference in the reaction of normal and castrated animals.

Changes in the urinary flow were inconsistent and not very marked.

The simultaneous injection of testicular and prostatic extracts.

No significant changes were produced when fractions Nos. 1, 2 or 4 were used (Table IV). In half of the experiments fraction No. 3 moderately increased the nitrogen metabolism up to a maximum of 19·9 % above normal. However, in the other half of the experiments on the injection of the same fraction the metabolism was slightly decreased or practically unchanged.

Table IV. *The influence of simultaneous subcutaneous injections of lipoid testicular and prostatic extracts Nos.* 1, 2, 3 *and* 4 *upon the nitrogen metabolism and urinary flow of rabbits.*

No. and description of rabbit	Total amount in g. per kg. and kind of extract injected			Percentage change in excretion of urine		Percentage change in nitrogen of urine		Change in nitrogen balance	
				Period of injection	Following control period	Period of injection	Following control period	Period of injection	Following control period
				%	%	%	%	g.	g.
15. Normal	0·13	$T_1+0·16$	P_1	+ 6·6	−10·4	− 4·2	− 7·6	+0·43	+0·78
25. Normal	0·16	$T_2+0·19$	P_2	+13·6	+26·2	+ 6·8	+ 5·1	−0·67	−0·50
19. Normal	0·08	$T_2+0·08$	P_2	+ 0·5	+ 2·8	− 0·7	+ 3·2	+0·06	−0·29
28. Normal	0·20	$T_3+0·08$	P_3	+15·3	−23·1	+ 8·7	+ 9·5	−0·77	−0·95
28. Normal	0·05	$T_3+0·10$	P_3	−14·1	−27·8	+15·0	+ 9·6	−1·20	−0·77
25. Normal	0·03	$T_2+0·05$	P_2	− 4·3	−30·4	− 6·0	+15·1	+0·59	−1·49
25. Normal	0·04	$T_2+0·10$	P_2	+ 6·7	+24·9	+10·0	+17·5	−0·66	−1·15
7. Normal	0·09	$T_3+0·09$	P_3	+74·0	−34·2	+19·9	− 4·5	−1·65	+0·30
19. Normal	0·14	$T_3+0·14$	P_3	− 6·1	− 6·7	− 2·3	− 8·7	+0·21	+0·81
15. Normal	0·14	$T_3+0·14$	P_3	− 9·6	−15·0	− 3·0	− 9·7	+0·27	+0·88
20. Normal	0·14	$T_3+0·14$	P_3	− 7·0	−18·4	−11·1	−12·9	+0·81	+0·94
1. Castrated	0·14	$T_3+0·14$	P_3	− 4·6	− 9·7	+ 2·8	− 6·5	−0·22	+0·05
2. Thyroidect. and castrated	0·14	$T_3+0·14$	P_3	+ 0·0	+ 8·3	− 5·5	−25·4	+0·34	+1·55
9. Thyroidect.	0·10	$T_4+0·09$	P_4	+28·5	+25·6	+ 1·3	− 5·5	−0·11	+0·46

To explain these divergent results the following suggestion may be considered. Rabbits 1, 15, 19 and 20, about 1 month before the present investigation, were used in an investigation on the influence of another extract of sexual glands and they failed to show an increase in nitrogen metabolism after the injection of T_3+P_3.

Rabbits 7, 25 and 28, injected with the same mixture, were either new rabbits, used for this investigation only, or at least 2 months had elapsed since the previous experiment with them. In these nitrogen metabolism increased in every case. The number of these rabbits is small for definite conclusions. However, the difference in the reaction to the injections of both these groups of rabbits may indicate the appearance in them after about 1 month of an insensibility to a second injection. This insensibility may later disappear.

However, taking into consideration the comparatively small number of the experiments, the divergent results mentioned obtained after the injection of $T_3 + P_3$ may be explained by the *individual* reaction of the various animals. Previously [Korenchevsky, 1925, 1, 2] it has been shown that castration or injection of emulsions of sexual glands gives results varying in degree. The explanations given (see pp. 169–171) might be equally applied in this case. The changes in nitrogen metabolism of rabbits 25 and 28 are especially notable as regards the respective activity of the different fractions (injected singly or as a mixture) of testes and prostate (Tables II, III, IV). The results obtained with *thyroidectomised* rabbits must be considered quite separately. In our previous experiments [Korenchevsky and Carr, 1925, 2] we found that in thyroidectomised animals the injections of the emulsions of the sexual glands caused no change or a decrease of the nitrogen metabolism. This is also true in the case of all the extracts used in the present investigation (see rabbits 2 and 8, Table II, and rabbits 2 and 9, Table IV). The *urinary flow* after the injection of a mixture of the different fractions was in some experiments much changed, as for example in the experiment on rabbit No. 7 (Tables I and II), when the increase was 74 % above normal. The divergence, however, in the volume of urine excreted in the other experiments was so great that it is impossible to draw any definite conclusions. The individuality of the animal seems to play an important part in the changes of urinary flow. The importance of individuality was strikingly emphasised in a recently published investigation on the ovarian hormone [Coward and Burn, 1927].

Summary.

1. A method is described for the isolation of lipoid fractions of the prostate and testis, which were effective in raising the nitrogen metabolism.

2. Whilst these fractions (T_3 and P_3) were little, if at all, active when injected separately, when injected simultaneously they increased the nitrogen metabolism of some rabbits, sometimes as much as 20 %.

3. This increase in the nitrogen metabolism was obtained in only half of the experiments. A possible explanation is suggested.

4. Although the changes of urinary flow were in some experiments considerable, they were of so inconsistent a nature that no conclusions are drawn.

5. None of the fractions isolated from the prostate or testes, injected singly or mixed in the doses given, is able to prevent the atrophy of the penis, seminal vesicles and prostate of castrated rats, or is able to produce hypertrophy of the genital organs of normal rats.

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LXIV. THE SEXUAL GLANDS AND METABOLISM.

VI. THE INFLUENCE OF WATER-SOLUBLE TESTICULAR AND PROSTATIC EXTRACTS FRACTIONATED AT VARIOUS ISOELECTRIC POINTS UPON THE NITROGEN METABOLISM OF RABBITS AND THE DEVELOPMENT OF THE GENITAL ORGANS OF RATS.

By VLADIMIR KORENCHEVSKY
and MARJORIE SCHULTESS-YOUNG.

From the Lister Institute, London.

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Historical.

We do not know of any work published on the subject which is mentioned above. An attempt to find the male sexual hormones among the lipoid fractions of the testis and prostate formed the subject of the previous paper [Korenchevsky, 1928]. The results which were obtained were not conclusive. The simultaneous injection of a particular fraction from the testis and from the prostate did sometimes increase the nitrogen metabolism, but never the growth and development of the male genital organs. The present paper deals with the results obtained by injecting various fractions of the water-soluble constituents of the sexual glands. In experiments on dogs, Korenchevsky and Bogoslovsky [1921] found that simultaneous injections of emulsions of prostate and testis produced a much greater effect upon the nitrogen metabolism than either separately. It was therefore suggested that the prostate has a function of inner secretion *and is in a synergetic relation* with the inner-secreting tissue of the testis. Later, Korenchevsky and Carr [1925, 1], in experiments on rabbits, showed that homologous prostatic emulsions alone increased the nitrogen metabolism and that the simultaneous injection of prostatic and testicular emulsions did not give a larger effect. This was ascribed to the fact that in the testis of the rabbit there are substances which depress nitrogen metabolism.

Hunt [1925] pointed out that in human patients the symptoms of deficient inner secretion of the testes are only re-established in a certain percentage of cases following successful transplantation of this gland. This indicates that the testicular hormone is not entirely responsible for the size of the sexual organs, their functional activity, the general mental and physical development, growth

of the hair, etc. As clinical cases of disturbance of the prostate often occur with the onset of impotence, Hunt combined testicular transplantation with treatment with a prostatic preparation. Oral administration of prostate tablets was without effect, but the effects of hypodermic injections of prostate extracts, prepared in the laboratories of Lilly and Co., have, he says, "been rather remarkable" and in each case a return of sexual function was stated to occur. These facts explain why, in the present investigation, the greater number of experiments was performed to study the influence of simultaneous injections of testicular and prostatic extracts.

Experimental.

In these experiments, for the purpose of testing the activity of the extracts, 51 rats and 18 rabbits were used. In selecting a method it seemed essential to choose one which would avoid the presence of insulin-like substances in the final products, for, as Korenchevsky and Carr [1925, 2] have shown, these may mask the effects of sexual hormones, since the latter seem to have weak and slow action as compared with that of insulin, which belongs to the group of strong and quickly acting hormones. In order to remove these substances the crude fraction was extracted with 80 % alcohol containing 2 % HCl.

Bull's or pig's testes and prostates were used for the isolation of the extracts, no difference being found between the extracts from the glands of these two species of animal.

The first stages of the method were similar to those of the method used by Collip [1925] for the extraction of the parathyroid hormone. The prostate or testis was minced in a Latapie's machine, mixed with an equal volume of 5 % HCl and left in the cold store for 2 days. The p_H, after diluting the mixture with an equal volume of distilled water, was then adjusted to 7·6–8·0 with weak NaOH solution. The material was then centrifuged, the liquid poured off and the residue re-extracted at p_H 7·6 and again centrifuged. The two supernatant fluids were united, made acid to Congo red and saturated with sodium chloride, the precipitate was filtered on a Büchner funnel and ground in a mortar with 80 % alcohol containing 2 % HCl, in order to extract the insulin-like substances. The material was centrifuged and re-extracted twice with 80 % neutral alcohol and again centrifuged. In some experiments sodium carbonate was used in place of hydrochloric acid [Dudley and Starling, 1924]. No difference was obtained in the results. The supernatant fluids were thrown away and the residue emulsified in distilled water and adjusted to p_H 7·6–7·8, at which point it is soluble. By adjusting the solution to p_H 6·0, a first precipitate was formed, which was obtained by centrifuging the material. A few preliminary experiments showed this to be inactive and it was therefore rejected. Large precipitates were obtained between p_H 5·4 and 4·8. These were obtained by centrifuging. The resulting fluid did not, except in a few cases, give any precipitate at p_H lower than 4·8. In a few cases a small precipitate was obtained at p_H 4·0. The usual process was therefore, after centrifuging off the precipitate

obtained at p_H 4·8 and if no precipitate was formed at p_H 4·0, to adjust to about p_H 3·0 with HCl and to precipitate the substances remaining in the liquid by saturating a second time with NaCl. In every case a very large precipitate was obtained and was filtered off on a Büchner funnel.

All the precipitates obtained as described above were dried in a vacuum over phosphorus pentoxide and when dry were ground in a mortar and kept in bottles, in desiccators, in the cold store.

The extracts of testis obtained at p_H 4·0 and 4·8 or by the second NaCl saturation will in this paper be called *AT* 40, *AT* 48 and *AT* 2*S* respectively. For the analogous prostate extracts the letter *P* will replace the letter *T*.

In those cases where the extracts were prepared in the same manner, but without extracting the insulin-like material with alcohol, the letter *A* will be omitted; for example, such a testis extract obtained at p_H 4·8 would be called "*T* 48."

A second method of fractionating was also tried. The crude extracts were converted into the hydrochlorides, using Dudley and Starling's insulin method [1924]. For this procedure extracts were used which had not been treated with alcohol. Only a part of the extract was found to form a hydrochloride soluble in acid alcohol. The soluble hydrochloride and the insoluble residue were both collected, dried and tested separately. Thus from all the extracts two fractions were obtained, a soluble hydrochloride and an insoluble residue. In these cases, to the abbreviations used above will be added the letters HCl; for example, *T* 48-HCl will be the hydrochloride of a testicular extract precipitated at p_H 4·8.

For the injections the dried extracts were ground in a mortar with a few cc. of water and adjusted to p_H 7·6–7·8. In about half of the experiments the extracts were subcutaneously injected in this form. Owing to the fact that the extracts, thus prepared, contained some insoluble matter, indurations were in some cases produced under the skin. These disappeared in about 1 or 2 weeks after the injections. In order to avoid this complication, for the rest of the experiments, the redissolved extracts were centrifuged in small sterile tubes and only the clear supernatant fluid was injected. In these no indurations or any other local reactions were noticed.

Analysis has shown that the crude extracts obtained at p_H 5·4 or 4·8 contained only about 28 % of organic soluble substances and those obtained after the second sodium chloride saturation about 96 % of soluble substances, of which about 17 % were inorganic salts (almost entirely NaCl).

The effect produced by a given weight of the extracts upon the metabolism was the same irrespective of whether the insoluble substances were, or were not, present in the injected extract. The amount of the extract injected was usually 0·03 g. per kg. per day, but in some experiments was 0·1 g. per kg. per day. The variation in the effect produced with varying doses was small.

The technique of the experiments in all other respects was exactly the same as that described by Korenchevsky [1925, 3] and Korenchevsky and Carr [1925, 1].

The influence of the extracts on the growth and development of the genital organs of castrated rats.

Experiments were performed on 51 rats, of which 17 were controls, the remaining being injected with the various extracts mentioned above. The single dose was from 25 to 50 mg. A total of twelve injections was made during the 30 days of the experiment. At the end of the experiment no difference could be detected between the penis, seminal vesicles and prostate of the injected rats and those of the control.

Influence of the extracts on the nitrogen metabolism of rabbits.

Altogether 120 nitrogen metabolism experiments were performed on 18 rabbits, of which 10 were normal, 4 castrated, 3 parathyroidectomised and 1 thyroidectomised.

In order to shorten the record of the results, only those experiments are included in the tables in which the metabolic changes exceeded 5 % above or below normal during the period of injection, or the following period. For the same reason detailed figures of the metabolism experiments are omitted. In the tables only the percentage changes in the nitrogen excretion of the urine and the changes of the nitrogen balance in grams during the period of injection and one of the following control periods are stated. In this latter the figures are those of the period of greater change. In discussing the results obtained with each extract the number of experiments which have been omitted will be given.

1. *Testicular extracts.*

Extract T 48 *precipitated from the alkaline solution at* p_H 4·8. This extract was tested in the amount of 0·1 g. per kg. of rabbit. Three experiments were performed, in one of which no change was obtained and in two others (Nos. 9 and 10, Table I) a slight increase. There was no marked change in urinary flow. This fraction might be expected to contain insulin-like substances. In order to remove them a previous extraction of the testicular tissue with alcohol had been carried out, in the way described above.

Extract AT 48 *precipitated at* p_H 4·8 *from an alkaline solution of the testicular substances which had previously been treated with alcohol.* In all the experiments (Nos. 11 to 19, Table I), with the exception of No. 12, the nitrogen metabolism was markedly increased during the period of injection in No. 11 to 14 % and in No. 18 to 25 % above normal. In the control periods following the period of injection a decrease of nitrogen metabolism was found in all the experiments except No. 11. In some cases the amount of this decrease was higher than that of the increase of the metabolism (Nos. 12, 14, 16), which may indicate the incomplete removal of substances which depress nitrogen metabolism. In four experiments with the hydrochlorides of this extract no significant change in metabolism occurred.

Table I. *Influence of the extracts T 40, P 40, T 40 + P 40, AT 48, T 48 and P 48 upon the nitrogen metabolism.*

No. of exp.	Extract	Percentage change in nitrogen of urine: Period of injection	Percentage change in nitrogen of urine: Following control period	Change in nitrogen balance g.: Period of injection	Change in nitrogen balance g.: Following control period
1	*T* 40	+17·4	+ 0·9	−0·30	−0·07
2		+ 9·9	−10·1	−0·75	+0·78
3	*P* 40	− 0·7	+17·5	+0·06	−1·48
4		+21·4	− 6·2	−1·58	+0·46
5	*T* 40 + *P* 40	+ 0·6	− 5·0	−0·23	+0·20
6		+28·6	− 6·7	−2·62	+0·12
7		+ 8·5	− 7·4	−0·67	+0·58
8		+ 1·1	+ 3·4	+0·41	+0·34
9	*T* 48	+ 7·6	−10·6	−0·40	+0·56
10		+ 1·7	+ 4·3	−0·13	−0·33
11	*AT* 48	+14·1	+12·5	—	—
12		+ 1·7	− 9·7	−0·13	+0·90
13		+ 9·3	− 6·0	−0·52	+0·34
14		+ 5·8	−11·2	−0·45	+0·87
15		+ 7·4	− 4·1	−0·56	+0·31
16		+ 6·4	−11·6	−0·52	+0·95
17		+ 6·8	− 3·5	−0·51	+0·26
18		+25·1	− 4·6	−1·71	+0·33
19		+ 9·6	− 4·4	−0·87	+0·40
20	*P* 48	+ 5·5	—	−0·24	—
21		+13·1	−17·2	−0·19	+2·33
22		+ 5·3	—	−0·21	—
23		+ 4·0	+ 5·5	−0·11	−0·23
24		+ 5·0	—	−0·34	—
25		+ 5·0	− 3·4	−0·32	+0·28
26		− 2·7	−10·8	+0·32	+1·27

Note. In all the tables the figures with + (increase) or − (decrease) denote the changes from the normal, *i.e.* the control period previous to the injections.

Table II. *Influence of the extracts AT 48 + P 48 and T 48 + P 48 upon the nitrogen metabolism.*

No. of exp.	Extract	Percentage change in nitrogen of urine: Period of injection	Percentage change in nitrogen of urine: Following control period	Change in nitrogen balance g.: Period of injection	Change in nitrogen balance g.: Following control period
27	*AT* 48 + *P* 48	+19·9	+ 7·9	−1·39	−0·69
28		+10·7	+ 4·9	−0·74	−0·30
29		+ 8·2	—	−0·63	—
30		− 0·1	− 8·3	−1·01	+0·66
31		− 0·5	− 8·7	+0·04	+0·75
32		+20·0	—	−1·25	—
33		+11·8	—	−0·94	—
34		+ 0·1	+ 7·7	+0·01	+0·13
35		+ 2·1	− 7·6	—	—
36		+12·2	—	−0·94	—
37		+ 5·2	+10·3	−0·32	+0·07
38		− 0·9	−11·5	+0·08	+1·05
39		+16·6	+14·6	−0·82	−0·69
40	*T* 48 + *P* 48	+12·1	+ 3·9	−0·75	−0·31
41		+ 3·1	−19·8	−0·57	+1·26

Extract T 40 precipitated at p_H 4·0 *after the removal of fractions precipitated at* p_H 4·8. Only two experiments were performed, the results obtained being similar to those obtained after the injection of *AT* 48 (see Nos. 1 and 2, Table I).

Extract AT 2S precipitated by saturation with NaCl *after removal of the extracts precipitated at* p_H 4·8 *and* p_H 4·0, *also the hydrochloride of AT 2S.* Twelve experiments were carried out with the extract *AT 2S*, in five of which no change in the nitrogen metabolism occurred. An increase was obtained in the remaining seven (Nos. 42 to 48, Table III). The degree and character of the increase were similar to those obtained after the injections of the two previous extracts. Changes in the urinary flow were irregular, an increase in eight experiments with a maximum of 51 % and a decrease in four experiments, with a diminution of 40 % in one instance, being obtained. An attempt to increase the activity and purity of this extract was made by converting the substances in the extract which would form a hydrochloride into this form. Altogether seven experiments were performed, but no improvement in the activity was obtained (Nos. 49 to 51, Table III); the remaining four showed even less change.

Table III. *Influence of the extracts T 2S, P 2S and their hydrochlorides upon the nitrogen metabolism.*

No. of exp.	Extract	Percentage change in nitrogen of urine: Period of injection	Percentage change in nitrogen of urine: Following control period	Change in nitrogen balance g.: Period of injection	Change in nitrogen balance g.: Following control period
42	*AT 2S*	+11·2	—	−0·75	—
43		+22·7	+ 7·1	−2·33	−0·54
44		+ 5·2	− 8·1	−0·40	+0·62
45		+11·7	+ 0·1	−0·52	+0·32
46		+ 9·2	− 8·1	−0·68	+0·65
47		+ 7·1	+ 2·9	−0·59	−0·24
48		+ 7·0	−10·2	−0·56	+0·81
49	*T 2S*-HCl	+13·9	+11·3	−1·64	−1·44
50		+ 3·1	−10·0	−0·27	+0·88
51		− 3·1	−11·0	+0·29	+2·06
52	*P 2S*	−18·6	− 5·4	+1·26	+0·41
53		+23·3	+21·6	−1·08	−0·99
54		+ 4·8	—	−0·03	—
55		+ 8·7	—	−0·09	—
56		+ 9·6	+14·4	−0·14	−0·41
57		+ 3·5	−11·7	−0·25	+0·82
58		+ 8·4	—	−0·65	—
59	*P 2S*-HCl	+13·9	+ 9·6	−1·31	−0·91
60		+19·1	+ 6·4	−1·78	−0·60
61		+ 9·2	—	−0·83	—
62		+12·4	—	−0·67	—

It was found during the process of converting the substances of the extract into the hydrochlorides that only a part of these were soluble in acid alcohol. The insoluble part was collected and dried separately but it was not found to

exert a significant influence on the nitrogen metabolism in the four experiments made. These experiments are omitted from the tables.

2. *Prostatic extracts.*

The fractions precipitated at p_H 4·8 and 4·0, and a third (*P 2S*), which was obtained by saturating the remaining solution with NaCl after the removal of the two fractions precipitated at their isoelectric points, and also the hydrochlorides of *P 2S*, were tested for physiological activity. Altogether 21 experiments were made, of which 20 are given in the tables (Nos. 3, 4, 20–26, Table I, and Nos. 52–62, Table III).

The influence of all these extracts upon the urinary flow was small and inconsistent. A decrease of nitrogen metabolism was observed in two only (Nos. 26, Table I, and 52, Table III). In the remaining experiments an increase during the period of injection or the following period was obtained, the maximum being 23·3 % above normal (No. 53, Table III). The hydrochlorides were no more efficient in their action (Nos. 59–62, Table III).

3. *The influence of simultaneous injection of testicular and prostatic extracts upon the nitrogen metabolism and urinary flow.*

The testicular extracts T 48, *AT* 48, *or AT* 48-HCl *simultaneously injected with the prostatic extract P* 48. Thirty-six experiments were made, in twenty-one of which no significant changes were obtained. The seven experiments with hydrochlorides were among the negative results, showing that the inactive substances of the precipitate at p_H 4·8 formed the hydrochloride. Four experiments were performed on thyroidectomised animals. The simultaneous injection of *P* 48 + *AT* 48 into animals without the thyroid produced no significant change in the nitrogen metabolism. The remaining experiments are given in Table II, from which it is clear that the simultaneous injection of these prostatic and testicular extracts produced an influence upon the nitrogen metabolism similar to that obtained by the injection of the separate extracts.

The changes in urinary flow were irregular, but sometimes large, 71 % above the normal in one experiment and 42 % below the normal in another. In twenty experiments out of thirty-six the change was not less than 20 % above or below normal. We have no definite explanation of these large and opposite effects of the injections.

The testicular extracts T 40, *AT 2S or T 2S*-HCl *simultaneously injected with the corresponding prostatic extracts P* 40, *P 2S or P 2S*-HCl *respectively.* Altogether fifty experiments were performed, five with the extracts precipitated at p_H 4·0, twenty with those precipitated by saturation with NaCl after the removal of the fractions precipitated at p_H 4·8 and p_H 4·0 and twenty-four on the hydrochlorides of *T 2S* and *P 2S*. In only twenty-eight experiments did the changes exceed the normal by 5 %. The figures of these experiments are given in Table I (Nos. 5–8) and in Table IV (Nos. 63–86).

Table IV. *Influence of the extracts AT 2S + P 2S and the hydrochlorides of T 2S + P 2S upon the nitrogen metabolism.*

No. of exp.	Extract	Percentage change in nitrogen of urine: Period of injection	Percentage change in nitrogen of urine: Following control period	Change in nitrogen balance g.: Period of injection	Change in nitrogen balance g.: Following control period
63	*AT 2S + P 2S*	+ 8·3	+ 1·4	+0·73	−0·12
64		+10·1	− 4·6	−0·59	+0·27
65		+14·2	+ 9·5	−1·57	−1·12
66		+29·2	+18·8	−3·20	−2·12
67		+12·4	—	−0·98	—
68		+13·6	+ 1·9	−0·94	−0·13
69		+11·9	—	−0·62	—
70		+ 6·5	− 3·6	−0·66	+0·19
71		+ 5·5	− 2·9	−0·17	+0·42
72		+10·7	− 3·7	−0·89	+0·31
73		+16·9	+22·2	−1·20	−1·57
74		+10·0	—	−0·82	—
75		+ 8·0	− 4·7	−1·12	+0·17
76		+12·7	−17·9	−1·03	+0·45
77		+ 7·2	−15·4	−0·69	+1·48
78		+ 6·7	−10·1	−0·70	+1·06
79		+ 7·7	− 9·4	+0·66	+0·81
80	*T 2S*-HCl + *P 2S*-HCl	+ 7·1	− 9·3	−0·71	+0·93
81		+ 7·9	− 3·4	−0·87	+0·53
82		+10·3	—	+1·23	—
83		− 3·2	−13·8	+0·34	+1·47
84		+10·9	+ 2·8	−1·00	−0·26
85		+ 5·7	—	+0·16	—
86		+11·6	− 3·2	−1·28	+0·35

The effects on the nitrogen metabolism of the simultaneous injection of the extracts precipitated at p_H 4·0 and those precipitated by saturation with NaCl (2*S*) were similar. In both cases the increase in nitrogen excretion was about the same, the proportion of animals in which no effect was observed being 20 %.

Table V. *The average changes in the excretion of nitrogen in the urine during the period of injection of various extracts and during the following control period in the experiments given in Tables I to IV.*

Extracts	No. of experiments used for average	Percentage change in nitrogen of urine (average): Period of injection	Percentage change in nitrogen of urine (average): Following control period
T 48	2	+ 4·7	−3·2
AT 48	9	+ 9·6	−4·7
T 40	2	+13·7	−4·6
AT 2S	7	+10·6	−2·7
T 2S-HCl	3	+ 4·6	−3·2
P 48	7	+ 5·0	−6·5
P 40	2	+10·4	+5·7
P 2S	7	+ 5·7	+4·7
P 2S-HCl	4	+11·2	+8·0
T 48 + *P* 48	2	+ 7·6	−8·0
AT 48 + *P* 48	13	+ 7·9	+1·0
T 40 + *P* 40	4	+ 9·7	−3·9
AT 2S + *P 2S*	17	+11·3	−1·3
T 2S-HCl + *P 2S*-HCl	7	+ 7·2	−5·4

Hydrochlorides prepared from the extracts 2*S* produced no changes in metabolism in about 71 % of the rabbits injected with this mixture. At the same time the average increase (Table V) of the nitrogen metabolism was less than was obtained with the original extract "2*S*," which had not been treated with HCl. Therefore the attempt to separate specific substances increasing nitrogen metabolism, by purifying the extracts by conversion into the hydrochlorides, was unsuccessful. The changes produced in the urinary flow were again inconsistent and ranged from 83 % above to 53 % below the normal, the figures in about 71 % of the experiments with 2*S* being below normal.

Discussion.

In the foregoing paper by Korenchevsky [1928] it was shown that in the case of the lipoids extracted from testicular and prostatic tissue fractions could be isolated from each, which were little, if at all, active alone, but which, when one fraction from the testis and one from the prostate were injected together, sometimes increased the nitrogen metabolism of rabbits as much as 20 %. This corroborates the hypothesis of synergetic correlation between testicular and prostatic glands, suggested by Korenchevsky and Bogoslovsky in 1921 on experimental evidence and by Hunt in 1925 on the basis of clinical observations. The lipoid fractions in Korenchevsky's experiments were, however, unable to prevent the atrophy of the penis, seminal vesicles and prostate of castrated animals, nor were they able to produce hypertrophy of the genital organs of normal rats. Until substances are isolated which are more consistent in their action on metabolism and can prevent the atrophy of the genital organs in castrated animals, an announcement of the discovery of the male sexual hormones cannot be justified. It is by no means necessary, however, that these two properties should be possessed by the one hormone.

An attempt was made to isolate the specific hormones as water-soluble protein or protein-like substances, these experiments being described in the present paper.

The results we obtained with these extracts were similar to those with the lipoids. At different p_H values, or by saturation with NaCl, fractions were obtained which stimulated nitrogen metabolism, but they were not able to stimulate the growth of the sexual organs of castrated or normal rats. As was the case with the lipoids, the action on metabolism of these isolated fractions was inconsistent, in some experiments being very slight or even absent or decreasing instead of increasing the metabolism.

As was the case with the lipoid fractions, a large part of this inconsistency in the results is explained by the incidence of an insensibility very quickly developing in many animals after the first injection. In the following table (Table VI), a few examples are given confirming this conclusion. A mixture of the extracts *A* 48 or 2*S* was injected.

An increase in the dose does not generally make any difference. For example, rabbit 38 Table VI) received at the second injection twice the dose

Table VI. *The influence of repeated injections.*

No. of rabbit	Percentage change in nitrogen of urine		No. of days between first and second injection
	First injection	Second injection	
37	+12·4	−5·7	1
38	+11·9	+6·5	1
34	+ 8·2	−4·2	1
1	+20·6	+3·5	1
1	+11·8	+0·1	1
19	+ 8·2	+0·2	1

given at the first injection. When the time between the following injections is more than 3 weeks, in some animals, the insensibility is less marked. For example, in the above table (Table VI) between the first and second experiment on rabbit 1 a period of 22 days of rest was given, although in both experiments the insensibility on the second day is very marked. However, even if all the experiments in which insensibility occurred are excluded, there remains a number of experiments in which only slight changes in the metabolism were obtained after the injections. Another possible explanation of the inconsistency of the results, suggested by Korenchevsky [1925, 1, p. 31; 2, p. 171; Korenchevsky and Carr, 1925, 1, p. 81] is as follows. The nitrogen metabolism is controlled in the living organism by different mechanisms, among which the inner secretion of the sexual glands plays an important, but not vitally necessary, part. The varying effects upon the metabolism obtained by the injection of different *impure* preparations of sexual glands may possibly be explained (1) by the presence in these preparations of non-specific principles (*e.g.* insulin-like substances); (2) by the varying degree of functional efficiency and therefore the varying reaction of the synergetic and antagonistic endocrine glands which are influenced by the injections. Coward and Burn [1927] noticed the remarkable fact that the individuality of the animals also plays a great part in their reaction to the injections of ovarian hormone. In their experiments on rats and mice the variation in the active unit of the oestrus-producing hormone was sometimes as great as 1000 %. From the point of view of this hypothesis all the preparations, lipoid [Korenchevsky, 1928], and water-soluble described in the present paper must be considered to be impure. Probably the removal of insulin-like substances caused the removal of some part, perhaps a considerable part, of the specific hormones, the avoidance of one difficulty producing another. It may be that some of the procedures used are destructive to the male sexual hormones. These hormones may be very unstable or only stored in the glands to a trifling extent. In any case in comparison with the ease with which powerful female sexual fractions can be isolated from the ovaries *the difficulty of preparing specific and powerful fractions from the testes must be emphasised.*

From private information we know that, besides the results mentioned in the historical review of the present paper, many others were unpublished, because the results obtained were entirely negative.

Doubt as to the existence of the specific male sexual hormones cannot be entertained, since the following facts are well established.

(1) Atrophy of the prostate, seminal vesicles and penis follows castration.

(2) This atrophy can be prevented by a transplantation of testis into the castrated animal.

Therefore the inner secretion of the male sexual hormone does exist and its specific hormones will no doubt be found sooner or later. A comparison of the activity of the different extracts, prepared by us, leads to the conclusion that the precipitates obtained at p_H 4·0 and by a final salting out, 2*S*, are the most active in stimulating the nitrogen metabolism (Table V). With the exception of prostate hydrochlorides, *P* 2*S*-HCl, all the hydrochlorides are definitely less active than the original crude extracts from which they were prepared. By the hydrochloride method probably those substances are isolated which depress the nitrogen metabolism, as is done by insulin-like substances. The exception of *P* 2*S*-HCl is probably due to the fact that the prostate does not contain a large amount of depressing substances, as has been shown previously [Korenchevsky and Carr, 1925, 2].

A comparison of the lipoid extracts [Korenchevsky, 1928] with the water-soluble extracts *P* 40 and 2*S* shows the latter to be stronger in their action on the metabolism. A comparison of the respective tables in the two papers shows this fact definitely.

It is interesting *that the presence of substances stimulating nitrogen metabolism was found by us both in the lipoid and in the water-soluble protein-like fractions of the male sexual glands.* The same fact was stated for the ovarian hormone, for Doisy and co-workers [1924] extracted the ovarian hormone by lipoid solvents and Laqueur and his co-workers [1925, 1926, 1927], and Dickens, Dodds and Brinkworth [1927] by their method, finally obtained a powerful extract, in water, of the ovarian hormone. It seems that the male and female hormones are similar in this respect.

Our attempts to isolate active fractions we consider have failed in several respects. Yet the whole series of investigations of Korenchevsky and his co-workers [1914–1928] on sexual glands and metabolism demonstrates the influence of sexual glands in stimulating nitrogen metabolism and that this property is possessed by some of the fractions isolated.

At the present moment we are approaching the problem of isolation of the male sexual hormones from an entirely different angle, the results of which seem to be encouraging. These results we hope to publish shortly.

In conclusion, we summarise the main results of the experiments in this and our other papers of the series.

General summary.

(1) Up to the present time we have been unsuccessful in our attempts to prevent the atrophy of the genital organs of castrated animals by means of

feeding with fresh or dried testicular tissue, or by injecting fresh emulsions, or different extracts, prepared from *normal* testis.

(2) In the same experiments prostatic tissue separately, or ingested simultaneously with testicular tissue, also failed to stimulate the genital organs of castrated animals, as was similarly the case with injections of the prostatic extracts used separately or simultaneously with the corresponding testicular extracts.

(3) Taking into consideration the comparative ease with which powerful sexual extracts can be isolated from the ovaries, the great difficulty in preparing specific and active fractions from male sexual glands must be emphasised.

(4) Lipoid fractions prepared by us from testis, by the same method as that used for the preparation of an active ovarian extract, did not produce any significant influence upon the growth of the genital organs or the nitrogen metabolism.

(5) One of the other lipoid fractions isolated, when injected simultaneously with the corresponding prostate fraction, stimulated the nitrogen metabolism to a moderate degree, thus supporting Korenchevsky's hypothesis of the presence in prostate of hormones which are synergetic to those of the testis.

(6) A method was described for the removal of depressing substances from the water-soluble extracts of bull's and boar's sexual glands and for the isolation of fractions which were able to raise the nitrogen metabolism of rabbits and rats to a moderate extent.

(7) The results indicate that of the water-soluble extracts of testis and prostate the more active are those precipitated at p_H 4·0, or obtained by a second salting out after the removal of the fractions previously precipitated.

(8) A simultaneous injection of these water-soluble testicular and prostatic extracts does not produce a stronger effect upon the nitrogen metabolism than is produced by the injection of one of the extracts.

(9) The effect of these extracts upon the urinary flow is sometimes very marked, but is on the whole inconsistent.

(10) There is no difference in the influence of these extracts upon normal, castrated or parathyroidectomised animals. Thyroidectomised animals have never shown any significant rise of nitrogen metabolism after these injections.

(11) The water-soluble extracts seem to be more active than the extracts soluble in lipoid solvents which we prepared. It is noteworthy that the ovarian hormone has been isolated both as lipoid and water-soluble substances.

(12) Neither the water-soluble, nor the lipoid fractions, which have been isolated, can be regarded as perfect preparations, since their action is often inconsistent. This is probably due to the presence in them of impurities, or to destruction of the active principles, or their loss with the rejected material, during the process of purification.

(13) Insensibility to a second injection of the extracts was noticed in several experiments. It can develop 24 hours after the first injection of water-soluble extracts. This may also explain some of the cases of inconsistent action of the extracts.

(14) Besides these two suggested causes of the inconsistent influence of the extracts isolated, the varying degree of functional state and therefore the varying reaction of the synergetic and antagonistic endocrine glands to the injections are also responsible for the cases of weak, or variable, effect of the extracts. Other investigators have also observed inconsistency in the action of ovarian hormone, possibly due to the same causes which have been already mentioned. Therefore this inconsistency appears to be common to both the male and female sexual hormones.

(15) The inner secretion of the sexual glands stimulates the whole organism. Therefore, theoretically the inner secretion would be expected to have a stimulating effect on metabolism. This suggestion is supported by the whole series of experiments of Korenchevsky and his co-workers [1914–1928] and by many other investigations, which have been discussed in the historical reviews of our publications.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We also wish to express our sincere gratitude to Sir Charles Martin for his continuous support and criticism of this work.

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LXV. A CRITICAL EXAMINATION OF METHODS OF EVALUATING VITAMIN A BY MEANS OF THE GROWTH OF RATS.

By ELEANOR MARGARET HUME
and HANNAH HENDERSON SMITH.

From the Department of Experimental Pathology, Lister Institute, London.

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When Steenbock and Nelson [1923] made the important deduction that the absence of vitamin D acts as a limiting factor in growth, it was clear that much of the past work, in which substances had been tested for their value in vitamin A by means of the growth of rats fed on a diet deficient in both the fat-soluble vitamins A and D, could no longer stand. Steenbock showed that the rat's reserve of vitamin A usually long outlasts that of vitamin D and that, for the first 5 or 6 weeks of its life on a diet deficient only in these two vitamins, growth is limited by the lack of vitamin D; if vitamin D is supplied, growth is restored, only to be limited again later when the rat has exhausted its supplies of vitamin A. Tests for vitamin A, which were in use before these facts were realised [cf. Zilva and Miura, 1921], must often have been made when the rat was deficient in vitamin D but still had a reserve of vitamin A, and a growth response which was really due to an addition of vitamin D would be ascribed to the action of vitamin A. Other tests may have been made when the rat had exhausted its reserves of both vitamins A and D; when this was the case the addition of a substance not containing them both would fail to restore growth and the substance would be returned as deficient in vitamin A, although it really might have contained it. It is impossible to interpret the old results, since knowledge does not exist as to the state of depletion of the rats which were then used, and one is forced to regard them all as dubious.

Sherman and Hessler [1927] maintain that their results are not affected in this way, because their experimental animals have always had a reserve of vitamin D which outlasted their reserve of vitamin A; this difference from Steenbock's animals they explain as being due to the fact that their stock animals receive milk while Steenbock's receive alfalfa. But Steenbock's theory was originally based on experiments made by one of the present writers [Hume, 1922] and by Goldblatt and Soames [1922] upon rats taken from a breeding stock which received milk in its diet. Indeed, it is safe to say that, at the Lister Institute, rats bred in the ordinary routine way have not been encountered whose behaviour suggested that their reserve of vitamin D was greater

than that of vitamin A. It is therefore very unsafe to rely upon any rat having such a constitution. Sherman and Hessler admit that, in spite of the peculiar constitution of their rats, it is probably wiser to supply vitamin D, when testing for vitamin A, and it is now generally recognised that this should be done.

Since Steenbock's discovery, he and a number of others have devised and used various ways of supplying vitamin D to the rats used in assaying vitamin A. In 1924 [Steenbock, Nelson and Black, 1924] he published his first paper, dealing with the actual technique of the determination of vitamin A. He administered vitamin D in the form of direct irradiation of the rat for 10 minutes daily. The irradiation was given from the start of the experiment, as soon as the rats were placed on the basal diet, and so were the various doses of alfalfa to be tested. The paper does not, however, go much beyond establishing the theory of the method. For one sample of alfalfa, it is seen that normal growth was established when the alfalfa was added as 4 %, 2 % and 0·98 % of the diet, but no dose is shown which yielded subnormal growth. On the other hand, a sample of weathered alfalfa gave no growth superior to that of controls when added to the diet in the same three percentages.

The writers, wishing to make a careful quantitative estimation of the vitamin A content of two samples of spinach, modified Steenbock's technique somewhat and devised the one used by Chick and Roscoe [1926, 2] for estimating the value of some different samples of milk. The modification consisted in feeding the rats for some weeks on a diet deficient in both vitamins D and A, and then giving vitamin D alone for a short period, until weight began to decline, and at that point adding the substance to be tested for vitamin A. For various reasons it was thought that this modified method would have advantages over Steenbock's original one. In the method as used by Chick and Roscoe, vitamin D was supplied by direct irradiation in most of the experiments, but in some of them it was provided by irradiating the hardened cottonseed oil of the basal diet. The latter modification was, however, not in use at the time when the tests on spinach, about to be described, were made; it was only introduced later as the result of those tests.

In the tests on spinach, three doses, 0·2, 0·4 and 1·0 g., of spinach were given to groups of rats, which had ceased to grow and had begun to show a decline on a diet deficient in fat-soluble vitamins, and which were receiving daily irradiation with a mercury vapour quartz lamp. It was believed that when the spinach was added the rats would show a resumption of growth, proportional to the dose of spinach given, but this did not prove to be the case. Throughout the range of doses of two samples of spinach, no consistent difference in the behaviour of the rats was found. There were considerable individual and sex differences (cf. Fig. 1), but in almost all groups one or more rats died from respiratory trouble, and of the rest most showed normal growth for a time, but then the curve flattened off before the rats had reached the weight at which growth normally slackens. A series of experiments was therefore set on foot

to find an explanation of this behaviour and to seek to ascertain how vitamin A might better be estimated. The resulting experiments extended over a considerable period, and while they have been in progress a number of other workers have published accounts of methods of testing for vitamin A and records of tests made. These are, however, more conveniently discussed after the present experiments have been described.

Experimental.

Young rats were used, bred at the Lister Institute, which were of 40 to 50 g. weight at the beginning of the experiment, and the usual precautions were taken with regard to sex and litter in their distribution amongst the experimental groups. Each rat was kept separately in a glass jar and bedded on sawdust.

The diet used throughout, except when modifications are especially described, was as follows:

Inactivated caseinogen	300 g.
Starch	750 g.
Hardened cottonseed oil ...	225 g.
Salt mixture	75 g.
Marmite	75 g.
Lemon juice	75 g.
Distilled water	900 cc.

The salt mixture was as follows:

Sodium chloride	51·9
Magnesium sulphate	164·0
Sodium dihydrogen phosphate ...	104·1
Dipotassium hydrogen phosphate	286·2
Calcium phosphate	162·0
Calcium lactate	390·0
Ferric citrate	35·4

Irradiation was carried out by means of a mercury vapour quartz lamp (Ulviarc), which had been in use for about a year. Rats were exposed to it for 10 minutes six times a week at a distance of about 50 cm. Hardened cottonseed oil was melted and exposed for 30 minutes at a distance of 33 cm. in a thin layer.

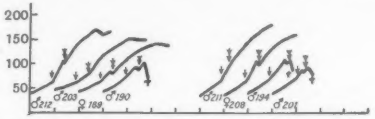
Series I. Experiments with spinach as source of vitamin A.

The object of the experiment was to test the value for vitamin A of the same samples of specially grown spinach as were tested for vitamin D by Chick and Roscoe [1926, 1]. The spinach (*Spinacea oleracea*) was that described by them (p. 146) as "summer-grown spinach, May—October, 1925," of which there were two kinds "open" and "frame" spinach. The "open" spinach was raised in the open ground; the "frame" spinach was grown in a well-ventilated

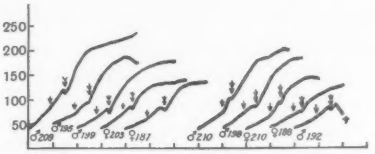
frame, glazed with Crookes's "A" glass. This glass has the property of absorbing ultra-violet radiations of wave-length shorter than 3780 Å., while transmitting 88 % of the visible light.

Three doses, 0·2, 0·4 and 1·0 g., of each kind of spinach were given daily, except on Sundays.

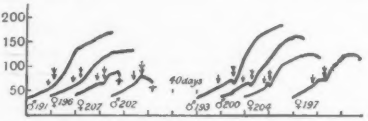
The rats received the diet deficient in both vitamins A and D for a period of from 35 to 40 days. Irradiation with the mercury vapour quartz lamp for 10 minutes daily was then applied and the growth rate at once increased, but after a short time again slackened or actually began to decline. The dose of spinach was then given daily with the result shown in Fig. 1. In all the groups,



Open spinach 0·2 g. Frame spinach 0·2 g.



Open spinach 0·4 g. Frame spinach 0·4 g.



Open spinach 1·0 g. Frame spinach 1·0 g.

↓ Vitamin D given as direct irradiation. ↓ Vitamin A given as doses of spinach.

Fig. 1. Weight curves of rats on a diet deficient in fat-soluble vitamins, vitamin D given as direct irradiation after 5 weeks. Doses of 0·2, 0·4 and 1·0 g. spinach, grown out-of-doors and under a frame, given daily 3 weeks later.

except those receiving 0·4 g. of "open" or 1·0 g. of "frame" spinach, one or two animals died, due to respiratory disease contracted before the spinach was administered, and these deaths must be discounted in evaluating the whole result. The surviving animals in the various groups grew normally for about 40 days after the beginning of the administration of the dose of spinach, but after that the growth curve began to flatten, regardless of the weight to which they had attained or of the dose of spinach which was being administered. No group of rats showed a consistently subnormal rate of growth immediately after the first administration of the spinach, and it might therefore be argued that none of the doses was minimal, but if that were so the question still remains, why the rats did not continue to grow normally until they were adult. As regards the vitamin A value of the two samples of spinach, it is fairly safe

to conclude that there was no great difference between them; had culture in the absence of ultra-violet light greatly reduced the content of vitamin A, this would have been revealed by the non-recovery of rats on the 0·2 g. dose of "frame" spinach. It is not, however, possible to draw a more exact quantitative conclusion. The result, as far as it goes, therefore, is in accordance with that of Coward [1927], who found that the short ultra-violet rays from a mercury vapour quartz lamp, used in conjunction with the visible rays, had no influence on the ultimate amounts of vitamin A contained in the plant tissues investigated.

After the rats had been receiving the spinach doses for some 10 weeks, various additions to the diet were made to see if restoration of growth could be brought about. In some instances these were successful, more particularly when 2·0 g. of wheat germ or 5 drops of cod-liver oil were given daily; additions of iodine, thyroid extract, irradiated cottonseed oil or oxidised cod-liver oil did not appear to confer any benefit.

On post-mortem examination most of the rats showed some degree of respiratory disease, the inception of which probably dated from the preliminary period on deficient diet, but which had also progressed during the period of spinach feeding. Many rats also showed abnormal eyes, suggestive of the early stages of xerophthalmia; even rats receiving a daily dose of 1·0 g. of spinach were so affected, but the effect was undoubtedly in part due to the daily exposure to the mercury vapour lamp.

As the results in testing the spinach were so unsatisfactory further experiments were planned to seek an explanation of them.

Series II. Experiments with butter as source of vitamin A.

In the next series of experiments butter was used as a more convenient source of vitamin A, since it could be stored and the same sample used throughout the experiment. New Zealand butter was chosen; since the cows are at pasture all the year round, it was thought that New Zealand butter was likely to furnish a material of high and tolerably constant vitamin A content. Doses of 0·2 and 0·5 g. daily were given.

It was thought possible that in the last series of experiments the rats had suffered from the constant daily exposure to the mercury vapour lamp. Some rats, therefore, in the series received direct irradiation, while others were given the hardened cottonseed oil of the basal diet, irradiated as already described. At first 5 drops of this were given to each animal individually, but it was soon clear that such an amount was insufficient, and after 10 days the whole of the hardened cottonseed oil (about 10 % of the diet) used in making up the basal diet was irradiated, and vitamin D was thus supplied in the basal diet. The amount supplied in this way was always found to be sufficient.

A series of rats was also included which received no additional vitamin D beyond that contained in a daily dose of 0·2 or 0·5 g. of butter.

It was thought possible that the long preliminary period on deficient diet without vitamins A or D might have a deleterious effect upon the health of the rat and that this injury might ultimately show itself in the failure of the rats to grow normally until they were adult; a comparison was therefore made between the growth of a series of rats kept for a time on the deficient diet and then given vitamin D and finally vitamin A, as in the previous experiment, and that of a series which received the basal diet together with vitamin D and the experimental dose of vitamin A from the very start of the experiment.

There were therefore 12 groups of rats, the weight curves of which are shown in Figs. 2 and 3, and which received treatment as follows:

Group 1. Irradiated cottonseed oil and 0·5 g. butter, from start of experiment
Group 2. " " " 0·2 g. " " " "
Group 3. Direct irradiation 0·5 g. " " " "
Group 4. " " 0·2 g. " " " "
Group 5. No addition of vitamin D 0·5 g. " " " "
Group 6. " " " 0·2 g. " " " "
Group 7. Preliminary depletion, then irradiated cottonseed oil, 0·5 g. butter
Group 8. " " " " " " 0·2 g. "
Group 9. Preliminary depletion, then direct irradiation, 0·5 g. butter
Group 10. " " " " " 0·2 g. "
Group 11. Preliminary depletion, no added vitamin D, 0·5 g. butter
Group 12. " " " " " 0·2 g. "

On reference to Figs. 2 and 3 and on comparing the growth in the groups which received vitamin D as irradiated cottonseed oil (1 and 2, Fig. 2, and 7 and 8, Fig. 3) with that in the groups which received it by means of direct irradiation (3 and 4, Fig. 2, and 9 and 10, Fig. 3), it is seen that there is no difference between the comparable sets which can be attributed to the different mode of administration of vitamin D. As in the case of the rats which received the various doses of spinach in Exp. 1, there was no quantitative difference in the growth of rats receiving daily doses of 0·2 or 0·5 g. butter, after a period of depletion (groups 7, 8, 9, 10, Fig. 3); the growth curve showed the same peculiar character with resumption of growth followed by a premature flattening, whether the rats were irradiated directly or received irradiated cottonseed oil. The flattening of the weight curve was therefore not due to over-irradiation. Leigh-Clare [1927] has also shown that rats suffer no ill-effect from prolonged irradiation if their eyes are shaded, and Goldblatt and Moritz [1926] showed that direct irradiation and irradiated fat are about equally good for supplying vitamin D to rats. The use of the irradiated cottonseed oil is, however, on the whole to be recommended as more convenient, since it does not bring about the abnormal condition of the eyes which direct irradiation does, and an accurate appraisement of the eye condition, as influenced by vitamin A deficiency, is therefore not interfered with. This method of supplying vitamin D was also preferred by Chick and Roscoe [1926, 2] in a part of their experiment to evaluate the vitamin A in milk for the same reason.

Of the rats receiving vitamin D from the start (groups 1, 2, 3 and 4, Fig. 2), though there was no difference between the weight curves of those receiving vitamin D as direct irradiation and those receiving it in the diet, yet there was

a definite difference between those receiving 0·2 g. of butter and those receiving 0·5 g. in either category. In each instance those receiving the smaller dose showed an earlier slackening of the rate of growth than did those to which the larger dose was given; in fact, when vitamins A and D were given from the beginning of the experiment, there were signs that the growth response of the rats varied quantitatively with the dose of vitamin A given, which it had failed to do when the experiment began with a period of depletion. Even so, the

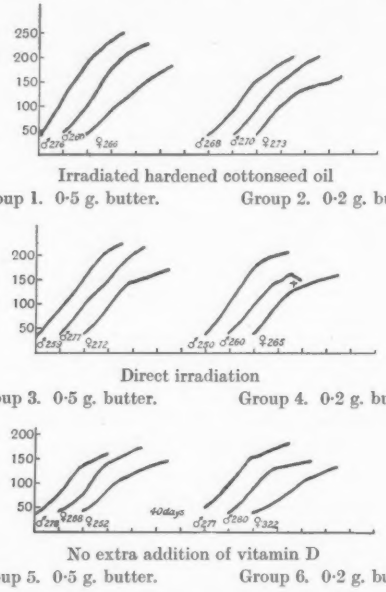
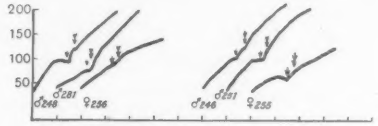


Fig. 2. Weight curves of rats receiving additions of 0·5 or 0·2 g. butter daily. Groups 1 and 2 received irradiated cottonseed oil as source of vitamin D. Groups 3 and 4 were irradiated directly and groups 5 and 6 received no extra addition of vitamin D.

growth of rats receiving 0·5 g. of butter daily was not fully normal, for the growth rate slackened prematurely, but not so early as did that of rats which received 0·2 g. of butter. It was, however, clear that in any further experiment there was more hope of obtaining a quantitative result when vitamins A and D were administered from the start than when they were administered after a period of depletion.

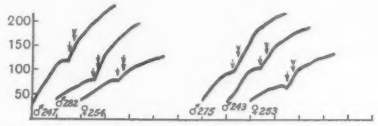
The explanation why quantitative results could not be obtained after a period of depletion was still not clear. The doses of butter given were evidently not sufficient to maintain normal growth after the rats' own reserves were exhausted, either because of an insufficiency of vitamin A or of some other factor. Yet those same doses of butter were able to restore normal growth for a time in rats already depleted of their reserves. The simplest explanation

would be that some other food factor was lacking and was acting, by its absence or inadequacy, as a limiting factor in growth. In the last series of experiments (Series I) some recovery had been obtained when wheat germ or cod-liver oil was added to the diet, and in the present series (Series II) increased butter and yeast extract seemed to have some beneficial effect, results pointing to an insufficiency of vitamin A, B or E in the diet. If the insufficiency were still one of vitamin A after the additions of butter had been made, the non-



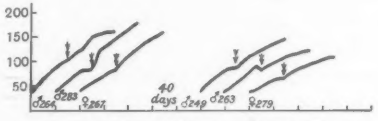
Irradiated hardened cottonseed oil

Group 7. 0·5 g. butter. Group 8. 0·2 g. butter.



Direct irradiation

Group 9. 0·5 g. butter. Group 10. 0·2 g. butter.



No extra addition of vitamin D

Group 11. 0·5 g. butter. Group 12. 0·2 g. butter.

↓ Vitamin D given. ↓ Vitamin A given as doses of butter.

Fig. 3. Weight curves of rats on a diet deficient in fat-soluble vitamins, receiving doses of 0·5 and 0·2 g. butter after a preliminary depletion period of about 9 weeks. Vitamin D given after the first 8 weeks as irradiated hardened cottonseed oil to groups 7 and 8, and as direct irradiation to groups 9 and 10. Groups 11 and 12 received no extra addition of vitamin D.

quantitative growth of the rats after a period of depletion still remained unexplained; this might, however, be due to a pathological condition initiated during the earlier period on deficient diet. Most of the rats at autopsy were found to have some degree of lung disease, and Macy and co-workers [1927, 1] have pointed out that when once a pathological condition of the upper part of the respiratory tract has been established by the use of a diet deficient in vitamin A it seems to be ineradicable, although the diet may be changed and the rat appear to thrive for a time. In the next series of experiments, therefore, the use of a preliminary period of depletion was abandoned.

The four remaining groups (5, 6, Fig. 2, and 11 and 12, Fig. 3) of the present experiment, in which 0·2 g. and 0·5 g. of butter were given from the start of

the experiment and after a depletion period, but without any addition of vitamin D, show how necessary it is that vitamin D should be specially given in all attempts to test for vitamin A. Groups 11 and 12, Fig. 3, reproduce the method of Zilva and Miura [1921]; in all four groups the growth is depressed, compared with that of groups which received corresponding doses of butter together with vitamin D. The insufficiency of vitamin D is therefore limiting the growth and obscuring any reaction which would be given by addition of vitamin A.

Series III. Experiments with butter and cod-liver oil as source of vitamin A and with doubled ration of vitamin B.

In the third series of experiments the various possibilities just sketched were examined and a special attempt was made to secure normal growth up to maturity in both sexes, as it was felt that no basal diet could be regarded as really satisfactory for the estimation of vitamin A unless it gave such growth when vitamin A in sufficient quantity was added as the sole addition.

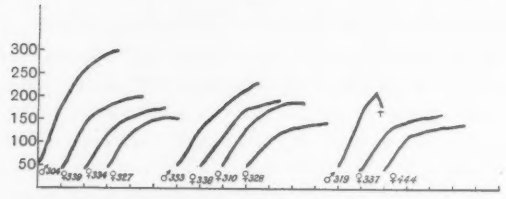
As has been stated, the use of a depletion period was abandoned and vitamins A and D were supplied throughout from the start of the experiment; irradiated cottonseed oil was again used as the source of vitamin D.

Groups of experiments were devised to test whether the failure of rats in the previous experiments to grow to maturity could have been due to insufficiency of vitamin A, B or E. To test the first possibility, a group receiving a daily dose of 1·0 g. butter was included, as well as groups receiving 0·5 and 0·1 g. To test the possibility of a deficiency of vitamin B, one series received a basal diet in which the amount of marmite had been doubled. To test the third possibility, groups were included which received 5 drops of cod-liver oil, and 0·5 g. butter together with 5 drops of cod-liver oil. While neither butter nor cod-liver oil is rich in vitamin E [Evans and Burr, 1927] yet butter is richer than cod-liver oil, which is almost devoid of it; if, therefore, growth were being limited by insufficiency of vitamin E and not of vitamin A the effect of administering cod-liver oil should be much inferior to that of administering butter.

The following groups were included:

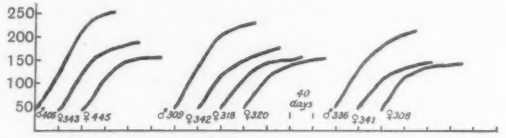
Group 13. Irradiated cottonseed oil from start and 1·0 g. butter
Group 14. " " " " " 0·5 g. "
Group 15. " " " " " 0·1 g. "
Group 16. " " " " 0·5 g. butter and 5 drops cod-liver oil
Group 17. " " " " and 5 drops cod-liver oil
Groups 18–22 as groups 13–17, but with double the amount of yeast extract in the basal diet

Figs. 4 and 5 show the results. In this series a large preponderance of females was used, only one animal in each group being a male. All through the present investigation it has been found much harder to produce continuously normal growth in females than in males; the weight curves of normally fed females, in any case, flatten off earlier than do those of males, so that there is a less range of growth over which differentiation of the doses can be displayed. For both these reasons females are therefore less suitable for an evaluation by



Irradiated cottonseed oil and double ration of vitamin B

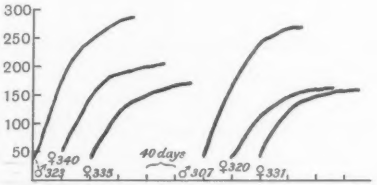
Group 18. 1·0 g. butter. Group 19. 0·5 g. butter. Group 20. 0·1 g. butter.



Irradiated cottonseed oil and single ration of vitamin B

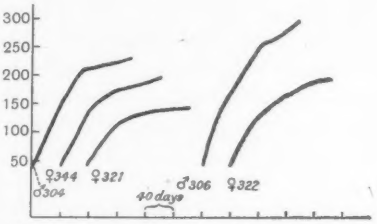
Group 13. 1·0 g. butter. Group 14. 0·5 g. butter. Group 15. 0·1 g. butter.

Fig. 4. Weight curves of rats on a diet containing irradiated hardened cottonseed oil as source of vitamin D, and receiving doses of 1·0, 0·5 and 0·1 g. butter from the start of the experiment. Groups 18, 19 and 20 received a double ration of vitamin B in the form of marmite.



Irradiated cottonseed oil and double ration of vitamin B

Group 21. 5 drops cod-liver oil + 0·5 g. butter. Group 22. 5 drops cottonseed oil.



Irradiated cottonseed oil and single ration vitamin B

Group 16. 5 drops cod-liver oil + 0·5 g. butter. Group 17. 5 drops cod-liver oil.

Fig. 5. Weight curves of rats on a diet containing irradiated hardened cottonseed oil as source of vitamin D, and receiving doses of 5 drops cod-liver oil, and 0·5 g. butter and 5 drops cod-liver oil. Groups 21 and 22 received a double ration of vitamin B.

growth. In the present experiments, however, it was felt that the end would not be satisfactorily attained unless normal growth could be evoked in females as well as in males by the addition of vitamin A to the basal diet.

On reference to Figs. 4 and 5 it is seen that on the whole the growth of the groups receiving the double doses of marmite is better than that of the groups receiving the single ration. The difference is not very great, but the only male in the whole series growing to 300 g. in the 5 months of the experiment (No. 304, group 18, Fig. 4), and the only females growing to 200 g. (No. 339, group 18, Fig. 4, and No. 340, group 21, Fig. 5) were in the double marmite series. This growth represents very good normal growth [cf. Macy *et al.* 1927, 1, Chart III]. The eyes of rats receiving the double marmite ration, together with high rations of vitamin A, also approached most closely to the normal. It was found very difficult to obtain rats with consistently perfectly normal eyes, by which is meant eyes that are fully exophthalmic and showing the blue line of the sclerotic all round the outside of the eyeball.

The three different doses of butter, whether combined with the single or the double marmite diet, gave growth results graded proportionally to the dose of butter given. The larger the dose, the longer was normal growth maintained. The individual variation in performance is great, but the general result is clear. Two of the excellent examples of growth referred to as occurring on the double marmite diet represented the growth of rats which were also receiving 1·0 g. of butter daily and were the best performances of any rats on the butter rations.

The behaviour of the rats which received cod-liver oil or butter and cod-liver oil (Fig. 5) was very uneven. Group 16, which received 5 drops of cod-liver oil and 0·5 g. of butter, added to the single marmite diet, was particularly poor, while the corresponding group, 17, which received 5 drops of cod-liver oil only, showed a good result. The latter group only contained two animals, but both were litter mates of two animals in group 16, so that the result must be taken as one of individual variation. Group 21, which received the double marmite ration, together with cod-liver oil and butter, showed an excellent result, approaching very closely that shown by the group which received double marmite and 1·0 g. butter daily and superior to that shown by the group which received double marmite and 0·5 g. butter. The cod-liver oil therefore seemed to be quite as effective in promoting growth as was the butter, and the beneficial effect of both must consequently be ascribed to their content of vitamin A and not, on the part of the butter, to its content of vitamin E. In any case it would appear, from the work of Evans and Burr [1927] and of Kennedy and Palmer [1926], that vitamin E should have been adequately supplied by the hardened cottonseed oil of the basal diet.

The general conclusion, therefore, to be drawn from the last series of experiments is that, when vitamin A is added in sufficient quantity (1·0 g. of butter daily) to the basal diet containing irradiated cottonseed oil, normal growth can be obtained in the rat when the addition is made from the start of the experiment. When doses less than sufficient are given, the weight curve flattens off after a period directly proportional to the size of the dose of vitamin A given. The individual variation in growth even among rats of the

same litter is, however, great and a clear-cut result cannot be expected when only a small number of animals is used. When an increased ration of vitamin B is given, the growth of a series of groups receiving different doses of vitamin A is transferred to a higher level, but the relation between the amounts of growth in the different groups is not altered. Growth is not so limited by the insufficiency of vitamin B in the diet used that the result, in attempting to evaluate vitamin A, is in any way obscured, but when the increase in vitamin B is made normal growth is more easily secured.

Discussion.

From their own work the writers were inclined to conclude that pathological processes, set on foot during the preliminary depletion period, were responsible for the premature slackening of growth which ensued when vitamin A had been administered for some weeks. But from a scrutiny of the work of others they concluded that this was not so.

In the present series of experiments 0·5 g. daily was the largest dose of butter administered after a depletion period, whereas it was found necessary to give 1·0 g. daily in order to produce normal growth without a depletion period. Macy *et al.* [1927, 2] mention that a larger dose of vitamin A is needed to produce normal growth after a depletion period than when the vitamin is given from the start of the experiment, and Steenbock and Coward [1927] were successful in obtaining normal growth when four yellow corn seeds daily were given after a depletion period. It would therefore appear that it is not impossible to obtain normal growth to maturity, after a depletion period, if a large enough dose of vitamin A is given, and had the writers given doses of 1·0 g. or more of butter daily, they would probably have obtained normal growth.

The question next arises, how can vitamin A best be evaluated? From the present three series of experiments the writers are inclined to recommend that tests for vitamin A should be made by giving a basal diet containing irradiated hardened cottonseed oil as the source of vitamin D, together with a range of doses of the substance to be tested for vitamin A, from the start of the experiment. The use of the double ration of vitamin B does not appear to be essential, as it simply serves to shift the whole series of weight curves to a slightly higher level, and to secure normal growth with a somewhat smaller dose of vitamin A than would otherwise be the case, as has just been explained.

All laboratories differ in the diet on which their rats are bred and, consequently, in the growth and behaviour of those rats when they are placed upon a deficient diet, so that it is not possible to lay down any universal rule that a certain growth performance should be correlated with a certain standard dose of vitamin A; but, for the rats which the writers are in the habit of using, they would regard the attainment of 300 g. by males and of 200 g. by females, at the age of 6 months, as an optimum performance which a sufficient dose of the material to be tested for vitamin A should be able to produce. This, however, demands an experimental period of 5 months, which is unpractically

long, but if lower doses are used a result would probably be apparent in 3 months. An early result is in any case impossible to attain, as the rats' own reserves may take from 6 to 8 weeks to exhaust, and before that has happened no quantitative result, on any system, can be looked for.

Where an early result is desired and the substance to be tested is believed to be deficient or wholly lacking in vitamin A, a purely qualitative result may be obtained by using a preliminary depletion period, and seeing whether the substance to be tested is capable of bringing about recovery after vitamin A deficiency has set in. If the dose given is wholly ineffective the rat dies, but if it contains vitamin A the rat recovers. This method was used by the writers [Hume, Smith and Smedley-MacLean, 1928] in testing yeast-fat, and the result was held to be a satisfactory proof that yeast-fat does not contain vitamin A. Drummond, Coward and Handy [1925], Moore [1927] and Rosenheim and Webster [1927] have used the method in this way.

Certain of the results of other workers show that by giving sufficiently small doses it is possible to obtain subnormal growth, graduated to the dose of vitamin A given, in the first few weeks after the depletion period, and before the premature flattening begins. Chick and Roscoe [1926, 2], in testing various samples of cow's milk by the same method as was used by the present writers in testing spinach, drew their conclusions only from the first 3 or 4 weeks of growth, after the first administration of the test doses of vitamin A. The growth during that period they found to be quantitatively related to the dose of vitamin A given, but they have stated (verbal communication) that after the first 3 or 4 weeks they found that growth always tended to slacken off, regardless of the dose of vitamin A given. No doubt they were confronted with the same phenomenon as were the present writers. 3 cc. was their highest dose of pasture milk and the rats receiving it showed normal growth for the first 4 weeks after depletion. Such a dose equalled about 0·15 g. of milk-fat, and the writers used 0·2 g. of pasture butter as their minimum dose after a depletion period and obtained normal growth initially; their results are therefore in agreement with those of Chick and Roscoe and none of their doses was small enough to show subnormal growth in the initial period after depletion. The writers did not follow up the possibilities of basing a method of testing vitamin A upon the varying growth in the first 3 or 4 weeks after the depletion period, as they became too much interested in investigating the cause of the premature flattening in the weight curve after that first 3 or 4 weeks.

Golding, Soames and Zilva [1926] obtained similar results to those obtained by Chick and Roscoe, using the same method. They tested butter from cows fed in various ways and obtained subnormal growth in the first 4 weeks after the depletion period, when as much as 0·6 g. of butter from cows on a poor diet was given daily to the rats. They only give summary curves of growth, but there is indication of premature flattening in some cases.

Amongst American workers, Steenbock, Nelson and Black's [1924] first outline of a technique has already been referred to, but it did not take definite

shape until 1927, when Steenbock and Coward [1927] published a full account of a method in which vitamin D was given from the start of the experiment, and the doses of material to be tested for vitamin A after a preliminary depletion period. They show curves of rats (p. 771) which grew to maturity, when four yellow corn seeds were given daily, after a period of depletion. In this and a subsequent paper by Coward [1927], a number of weight curves are set out of rats which received different doses of various cereals or germinated cereals. In a great many of them there was no recovery when the test material was given, showing that it was lacking in vitamin A; some of the curves show subnormal growth on small doses and some show early normal growth followed by premature flattening [Coward, 1927, Chart X, p. 795], such as the writers have described. It would almost appear from a study of these weight curves, and of the results obtained by the present writers and by Chick and Roscoe [1926, 2], that when a depletion period is used, and various doses of vitamin A covering a wide range are given, the growth response of the rat is as follows.

(1) With a very small dose of vitamin A, such as one yellow corn seed daily [Coward, 1927], there is no recovery and death ensues.

(2) With somewhat larger doses, such as two yellow corn seeds daily [Coward, 1927], or less than 0·1 g. pasture butter daily (present paper), or less than 3 cc. milk (0·15 g. milk-fat) from pasture cows [Chick and Roscoe, 1926, 2], there is recovery with subnormal growth, which is quantitatively related to the dose of vitamin A for the first few weeks, but later ceases to be so, the growth curve flattening off.

(3) With still larger doses, such as 0·5 g. pasture butter daily (present paper), 1·0 g. spinach daily (present paper), or two wheat shoots irradiated for some hours on 4 or 5 successive days [Coward, 1927], normal growth is produced in the first few weeks after the depletion period, but is followed by a premature flattening of the weight curve.

(4) With still larger doses, such as four yellow corn seeds daily [Coward, 1927] and perhaps a daily dose of 1·0 g. or more of pasture butter, though this dose was not tested by the present writers, after a depletion period, normal growth is obtained up to maturity.

When, however, vitamin A is given from the start of the experiment without any preliminary depletion of the animal's reserves, there is normal growth to maturity if the dose of vitamin A is large enough, and, if it is not large enough, the period for which normal growth is maintained is proportional to the amount of vitamin A given. This latter behaviour is easily intelligible, but it is not easy to understand why, after the rat's reserves have been exhausted, a certain dose of vitamin A should be able to evoke growth at a normal or a subnormal rate for a time, but not up to maturity. It might be that the daily supply needed is much larger when the rat is larger, or, much more probably, that the sudden remedying of a deficiency is met by the organism with a tremendous initial response which it is unable long to sustain.

Macy and co-workers [1927, 2], working on human milk, and Outhouse,

Macy and co-workers [1927], on cow's milk, show curves which fall into line with the above scheme. They include one series in which no depletion period was used and in which the growth on 1·5 cc. and 2 cc. of human milk was compared. On 2 cc. normal growth was produced up to maturity, but on 1·5 cc. normal growth ceased after about 8 weeks; the result given is clear and is comparable with that obtained by the writers when different doses of butter were given from the start of the experiment. When a depletion period was used, Macy found that larger doses of milk were needed to produce normal growth; there is then much greater variation between the individual curves and there is not a sharp differentiation between the doses. Most of her curves seem to be of the type in which normal growth occurs initially, followed by premature flattening, though with some of the larger doses normal growth takes place. These workers suggest a variety of other symptoms of vitamin A deficiency, the date of incidence of which might be used in the quantitative evaluation of vitamin A; such, for instance, are the appearance of xerophthalmia or of cornified cells in the vaginal smear, but to get a sharp result from the study of these symptoms would appear to the writers to be even harder than to get such evidence from a method of growth comparison.

The method of Sherman and Munsell, as employed by Sherman and Hessler [1927], has already been referred to. They regard the addition of vitamin D to the diet as perhaps unnecessary, but a wise precaution, and make their standard the restoration of growth to a rate of 3 g. weekly, after a depletion period. They show an estimation of vitamin A in carrot on these lines. They use a large number of animals, about two dozen in each group, but they only give figures for the average growth increment for one experimental period of 8 weeks. The data are thus insufficient for any analysis of the curves; presumably they are working with doses which are only just sufficient to bring about recovery and restore a very slow subnormal rate of growth. The same method was used by Quinn and co-workers [1927] to examine the vitamin A value of green plant tissue other than leaves, but they gave no vitamin D in any form, so that their results cannot be regarded as satisfactory.

Dye and co-workers [1927] made use of a method with a preliminary depletion period and irradiated basal diet. They give only average curves in which the sexes are not differentiated nor the range of variation of behaviour shown. With 0·3 g. of lettuce leaf daily their curve is of the type showing normal growth initially, after depletion, followed by premature slackening after 5 weeks. Supposing lettuce leaf to have somewhat the same content of vitamin A as spinach leaf, this result agrees with that obtained by the writers with spinach. Smaller doses or doses of less potent material gave subnormal growth with premature flattening of the curve.

Dutcher and co-workers [1927] used an irradiated basal ration, with a depletion period of 5 weeks, followed by 5 weeks' feeding of the test ration. They gave doses of from 1·0 to 3·0 cc. of variously treated milks, and got subnormal growth in varying degrees. They show no curves and only give average

figures in which sexes are not differentiated, so that the results cannot be analysed; but premature flattening might not be apparent in 5 weeks.

Supplee and Dow [1927] used a depletion period in estimating the vitamin A in milk and had groups of rats irradiated directly, receiving irradiated basal diet and receiving no addition of vitamin D beyond that contained in any milk supplement given. They obtained much better growth when milk supplements were given alone than when they were given with extra vitamin D in any form; they do not explain this curious result, which makes their work difficult of interpretation.

Bethke and co-workers [1927], testing vitamin A in various samples of egg-yolk, used a depletion period, with direct irradiation. They show a series of curves in which there is a great deal of individual variation. The growth responses for the different doses range from non-recovery, through recovery with subnormal growth, to recovery with normal growth. Some curves show signs of the beginning of premature flattening. A survey of the results of other workers thus seems to support the conclusions which the writers have drawn as to the possible types of growth response.

One of the really great difficulties in the present type of work lies in the individual variability in performance of the experimental animals, even when every known precaution is taken in breeding uniformly and in selecting litter mates. This difficulty is particularly stressed in the recent Report to the League of Nations on the biological assay of cod-liver oil [1928] and in an extension of the same piece of work by Leigh-Clare and Soames [1928]. The report deals with work from a number of laboratories and points out that even a moderate degree of accuracy can only be hoped for when a large number of animals is used, so as to eliminate the effect of the wide deviation of some individuals. The practical difficulties are thus still further enhanced by the need for large numbers of animals to be used. In that Report it is laid down by the Accessory Food Factors Committee (of the Medical Research Council and Lister Institute) that the method should include a preliminary depletion period, during the whole or part of which vitamin D should be given in the form of direct irradiation or of irradiated foodstuff. This method was adopted by the four laboratories who undertook to make the test independently. The laboratories were in tolerable, but by no means complete, agreement in their results, and both in the Report itself and in the extension of it by Leigh-Clare and Soames [1928] the want of accuracy in the results is stressed. No individual growth curves of rats are given, so that the individual variation in behaviour is not shown, but it is to this factor, as has already been mentioned, that the inaccuracy is ascribed. Difficulties such as troubled the writers when testing for vitamin A in spinach do not seem to have been encountered by these workers, nor indeed has any reference to them been found in the literature.

From the writers' own results and from an analysis of the work of others it would appear that the best hope in attempting an estimation for vitamin A is either to give the test dose from the start of the experiment and seek a dose

which produces a given amount of growth in a given time, taking growth to 300 g. for males and 200 g. for females at 6 months of age as that which an optimum dose ought to give, or to seek such doses as will, after a depletion period, give subnormal growth, graded to the dose, for the first few weeks. This latter method gives a very wide individual variation, the rats are in indifferent health as the result of the depletion period, and the danger exists that all the doses selected may lie within the range which produces normal growth followed by premature flattening. This range may be wide, including doses differing by 500 %, as the writers found in the case of spinach.

Summary.

(1) Experiments are described in which doses of 0·2 to 1·0 g. of two kinds of spinach were tested for vitamin A, by the growth of rats. A depletion period was used and vitamin D was given as direct irradiation. All doses of spinach gave normal growth curves for a time, followed by premature flattening and no quantitative result was obtained.

(2) Similar experiments were made with doses of 0·2 and 0·5 g. of butter, given with and without a depletion period, and with irradiated hardened cottonseed oil and direct irradiation as sources of vitamin D. There was no difference in the results yielded by the two sources of vitamin D and a normal growth curve, with premature flattening, was obtained with both doses of butter after a depletion period. Without a depletion period, the result was quantitative, normal growth being continued for a period directly proportional to the dose of vitamin A.

(3) Further experiments were made with doses of 0·1, 0·5 and 1·0 g. butter, and some with cod-liver oil, without a depletion period. One series received a double ration of vitamin B. Normal growth to maturity was obtained with 1·0 g. butter and the double ration of vitamin B, and the growth on 0·5 g. and 0·1 g. was proportionately less, according to the dose. The series on the single ration of vitamin B gave the same result but on a slightly lower level throughout. The results with cod-liver oil indicated that the only limiting factor in growth had been the amount of vitamin A and not any other dietary factor.

(4) It was concluded from the present experiments and from the results of other workers that the growth response of the rat to a series of doses of vitamin A after a depletion period is as follows. (*a*) Very small or no dose. No recovery. (*b*) Small doses. Subnormal growth, graded quantitatively to the dose; later, premature slackening. (*c*) Larger doses. Normal growth for a time, followed by premature slackening. (*d*) Optimal dose. Normal growth to maturity. The peculiar character of the growth under (*b*) and (*c*) is regarded as a disproportionate response to a supply of vitamin after depletion, which cannot be maintained subsequently.

(5) It is suggested that evaluation of vitamin A is best made by dispensing with the depletion period, or if it is used, as being quicker, by employing only doses which are likely to give the responses (*a*) and (*b*).

It is desired to express particular thanks to Sir Charles Martin for his help and advice, to the Lister Institute for the hospitality of its laboratories and to the Medical Research Council for grants to the workers.

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LXVI. THE ASSAY OF THE ANTIRACHITIC VITAMIN D.

By KATHARINE MARJORIE SOAMES and JOAN CLARE LEIGH-CLARE.

From the Department of Experimental Pathology, Lister Institute, London.

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In the past the methods for assay of vitamin D in foodstuffs have consisted usually in determining the power of the foodstuffs to prevent or cure rickets induced in rats by a diet containing unfavourable amounts, or an unfavourable balance, of calcium salts and phosphates. Such diets have been devised and used by McCollum and his co-workers [1921], by Sherman and Pappenheimer [1921] and by Steenbock and Black [1925].

When young rats are nourished on these diets for two or three weeks without any supplement they are in effect starved of calcium or of phosphorus or of both, and almost invariably develop a degree of rickets which can be recognised by typical histological appearances, and they have an abnormally low proportion of calcium in the bones. The rickets can be prevented or cured by any powerful antirachitic agent. Such are irradiation of the animal or its food with ultra-violet light, or the administration of cod-liver oil or irradiated ergosterol [Rosenheim and Webster, 1927]. In animals fed on low-phosphorus rachitogenic diets, these measures seem to result in diverting the inadequate supply of phosphorus to the bony tissues in preference to the soft ones [Chick and Roscoe, 1926, 1]. The animal does not grow, but calcium phosphate is laid down in the bones, and these show no marked divergence from the normal in structure. The amount of any supplementary foodstuff which is found necessary to prevent rickets on such a diet is in inverse proportion to its antirachitic value.

The above method does not, however, satisfy the primary requirement which should be demanded of any biological method for vitamin assay, viz. that the basal diet of the experimental animal, while devoid of the vitamin in question, should contain optimal amounts of all other necessary constituents. All the diets mentioned above, in addition to being devoid of vitamin D, contain a defective salt mixture and an inadequate provision of vitamin A: in some there is also an inadequate supply of protein and of vitamin B.

It is obvious that the basal diet should contain a perfectly balanced salt mixture, and adequate protein supply, as well as optimal amounts of vitamins A and B. The problem of finding such a perfect basal diet ultimately resolves

itself into a search for some suitable source of vitamin A which shall be devoid of vitamin D. For these two fat-soluble vitamins are usually found together in nature and although vitamin D is much less abundant there are few known sources of vitamin A in which traces of vitamin D cannot also be demonstrated.

Steenbock, Nelson and Black [1924] found that "hog millet," when given to the extent of 84 % of the diet, provided adequate vitamin A and did not add materially to the antirachitic value of the diet. They also investigated dried alfalfa but found that the action of vitamin D could be demonstrated if more than 1 % was present in the ration. Miss E. M. Hume, in the course of work not yet published and kindly placed at our disposal, used wheat embryo as a source of vitamin A for young rats. She found that a daily dose of 2 g. of wheat embryo provided no significant amount of vitamin D, as judged by the percentage of ash in the fat-extracted bones at the end of the experiment. The leg bones of control animals, receiving no addition to the experimental diet deprived of fat-soluble vitamins, had an ash content of from 50·9 to 55·3 %, while in those receiving the wheat embryo, this value varied from 48·5 to 52·9 %.

Criteria adopted.

(*a*) *Calcification of bones.* When young rats are fed upon diets which contain an adequate supply of calcium salts and phosphates, even when vitamin D is absent, it is not usual to obtain a degree of rickets that can be recognised histologically. Defective calcification of the bones is nevertheless shown by the occurrence of osteoporosis and by a lowered calcium content, which can be demonstrated by chemical analysis.

The effect on the bony tissues of diets containing optimal salt content, but deprived of fat soluble vitamins, has been studied by Chick, Korenchevsky and Roscoe [1926]. These authors came to the conclusion that the best measure of bone calcification was given by the value of the A/R ratio or the ratio of the amount of mineral ash to the amount of organic material contained in the fat-extracted bone. The value of this ratio in a normally nourished young rat was found to be about 1·5, and if the diet were deficient in vitamin D ("*F*" diet + fresh autumn-grown spinach, see their Table, p. 626) this value fell to about 0·9 to 1·0. When nourished on a low-phosphorus rachitogenic diet, the value of A/R can be as low as 0·5 to 0·3 [Chick and Roscoe, 1926, 2]. Histological rickets is prevented on such a diet by supplying vitamin D as cod-liver oil or by ultra-violet irradiation, but the calcification of the bones never approaches the normal level although A/R may be raised to a value of about 1·0.

(*b*) *Growth; increase in body weight.* Steenbock, Sell and Jones [1923] and Steenbock and Nelson [1923], were the first to insist that the antirachitic vitamin D was essential in a diet to secure normal increase in weight in young rats. Steenbock and his collaborators [1924] have shown further that increase in weight may be used as a criterion for vitamin D assay if the young animals

are provided with all other essential dietary factors, including vitamin A. These investigators have done service to this branch of nutritional study in maintaining that no single vitamin can properly be considered as an especial growth factor, seeing that under appropriate circumstances, growth can be limited or entirely prevented by deficiency of any one of a whole series of necessary constituents, including vitamins.

Diets.

The work described in the present paper resolves itself into an attempt to discover a satisfactory basal experimental diet which shall contain adequate vitamin A unaccompanied by vitamin D. In consideration of the results obtained by Steenbock and his co-workers and by Hume, the three materials selected for trial were wheat embryo, hog millet and alfalfa meal. The following four diets were prepared.

1. Diet *F*, a diet complete in all other respects but devoid of both fat-soluble vitamins A and D.

Diets 2, 3 and 4 in which vitamin A was supplied respectively, as 20 % wheat embryo, 50 % hog millet, and 1 % alfalfa meal.

In Diets 2, 3 and 4 the amounts of starch and caseinogen were modified so that there should still be about 50 % carbohydrate and 20 % protein, reckoned on the dry weight of the diet.

Table I.

	Parts by weight			
	1. *F*	2	3	4
Wheat starch	50	43·7	14·2	49
Inactivated caseinogen	20	14·3	14·3	20
Cottonseed oil	15	12·4	13·3	15
Marmite	5	5·0	5·0	5
Salt mixture (McCollum No. 185)	5	5·0	5·0	5
Lemon juice	5	5·0	5·0	5
Wheat germ	—	20·0	—	—
Hog millet	—	—	50·0	—
Alfalfa meal	—	—	—	1
Distilled water	50	50·0	50·0	75

Results.

These diets were tested upon young rats about four weeks old, of 40–50 g. body weight. There were nine groups of such rats, each containing four animals. Group 1 received Diet *F* and may be regarded as the "negative control" group. The animals in Group 5, which supplied a series of "positive controls," also received Diet *F*, but in addition 20 mg. daily of cod-liver oil. Groups 2, 3 and 4 received, respectively, Diets 2, 3 and 4. The remaining four groups, viz. 1*R*, 2*R*, 3*R* and 4*R* received respectively, Diets *F*, 2, 3 and 4, but vitamin D was supplied in addition by means of irradiation of the hardened cottonseed oil contained in the diet. The oil was exposed for 30 minutes in a thin film at a distance of 35 cm. to the irradiation from a mercury vapour quartz lamp.

The 36 animals used for this test were derived from five different litters,

arranged as shown in Table II; they were maintained for 50 days on their respective diets. Table II shows the average increase in body weight and also the results of the chemical analysis of the leg bones (femora, tibiae and fibulae) at the end of this period. The proportion of calcium in the bones may be taken as equal to about 37 % of the total ash [see Chick, Korenchevsky and Roscoe, 1926].

The results show how disappointingly great is the variation between the individual rats in the various groups, both as regards increase in weight and the proportion of ash contained in the bones. This variation is sometimes greater than that occurring between the averages calculated for different groups.

Groups 2, 3 and 4 show an increased rate of growth compared with Group 1. It is difficult to decide to what extent this increase (41, 43, 33 g. in 50 days, in Groups 2, 3 and 4 respectively, compared with 25 g. in Group 1) is significant, having regard to the variation between individual rats in each group. The growth shown by the rats in Group 1 is an indication of the amount of the reserves of vitamin A and vitamin D possessed by the rats at the beginning of the experimental period and is limited by deficiency of both these vitamins. In Groups 2, 3 and 4 the extra provision of vitamin A in the diets may account for the small extra amount of growth. That this growth was limited by deficiency of vitamin D only and that diets containing 20 % wheat embryo and 50 % hog millet contain abundant vitamin A, while devoid of vitamin D, is indicated by the following facts. The rats in Groups 2 and 3 have no greater proportion of ash in their bones (% ash in fat-extracted bones, 46·7 and 48·1; $A/R = 0{\cdot}88$ and 0·93 respectively) than the rats in the control Group 1 (% ash in fat-extracted bones 49·1; $A/R = 0{\cdot}97$). When, however, abundant vitamin D is provided, as in Groups $2R$ and $3R$, both growth and bone calcification become optimal.

The results also suggest that 1 % alfalfa meal provides slightly inadequate vitamin A, and contains traces of vitamin D. The bone calcification (51·7 % ash) of the rats in Group 4 is, on the average, slightly better than in the control Group 1 (49·1 %). On the other hand, the average increase in body weight during the experimental period (33 g.), while greater than that of rats in Group 1 (25·2 g.), is less than that of those in Groups 2 and 3 (41·5 g. and 42·7 g. respectively).

The rats in Group 5, which received 20 mg. daily of a cod-liver oil which has been in use for some time in this Institute, were intended to serve as "positive control" animals. The results obtained, however, suggest that this oil may have deteriorated as regards its content of vitamin A, and that the growth of the rats in this group may have been limited slightly in consequence. The average growth, 12 g. weekly, though not markedly subnormal, is certainly lower than in Groups $2R$, $3R$ and $4R$. That the vitamin D supplied in 20 mg. daily of this oil was, however, adequate is shown by the values of the ash content of the fat-extracted bones (60·2 %), and of the A/R ratio (1·54), which are both up to normal value.

Table II. *Growth in weight and bone calcification of young rats maintained for* 50 *days on diets (a) devoid of vitamins A and D, (b) containing vitamin A as supplied from various foodstuffs, and (c) with vitamin D in addition. The weights of the rats at the beginning of the experiment varied from* 36 *to* 52 *g.*

	Diet							Analysis of bones (femur, tibia and fibula)							
Group	Vitamin A	Vitamin D	Rat no.	Sex	Litter	Increase in body weight g.	Average increase g.	Dry weight of fat-extracted bones g.	Average dry weight of extracted bones g	Weight of ash g.	Average weight of ash g.	% ash in extracted bones g.	Average % ash in extracted bones g.	*A/R*	*A/R* Average
1	0	0	875	F	1	13		0·390		0·1774		45·5		0·83	
			893	F	3	4		0·281		0·1351		48·1		0·93	
			901	F	4	24		0·359		0·1882		52·4		1·10	
			911	M	5	60	25·2	0·635	0·416	0·3194	0·205	50·3	49·1	1·11	0·97
2	20 % wheat embryo	0	877	M	1	61		0·492		0·2271		45·1		0·82	
			885	F	2	27		0·405		0·1990		49·1		0·97	
			894	M	3	40		0·452		0·1959		43·3		0·77	
			902	F	4	38	41·5	0·405	0·438	0·2007	0·204	49·5	46·7	0·98	0·88
3	50 % hog millet	0	878	M	1	70		0·586		0·2776		47·4		0·90	
			886	F	2	41		0·483		0·2313		47·9		0·92	
			895	M	3	24		0·426		0·1928		45·3		0·83	
			903	F	4	36	42·7	0·428	0·481	0·2222	0·231	51·9	48·1	1·08	0·93
4	1 % alfalfa meal	0	879	M	1	37		0·500		0·2472		49·4		0·98	
			887	F	2	32		0·505		0·2628		52·0		1·09	
			896	M	3	42		0·532		0·2675		50·3		1·01	
			904	F	4	21	33·0	0·371	0·477	0·2048	0·245	55·2	51·7	1·23	1·08
5	Cod-liver oil		884	F	1	85		0·794		0·4851		61·1		1·57	
			892	F	2	77		0·823		0·5006		60·8		1·55	
			909	F	4	94	85·3	0·805	0·807	0·4843	0·490	60·2	60·7	1·51	1·54
1 *R*	0	Irradiated cotton-seed oil	883	F	1	28		0·422		0·2351		55·7		1·26	
			908	F	4	56		0·639		0·3921		61·4		1·59	
			913	M	5	99		0·867		0·5067		58·4		1·41	
			914	M	5	102	71·2	0·910	0·709	0·5380	0·418	59·1	58·7	1·44	1·42
2 *R*	20 % wheat embryo	Irradiated cotton-seed oil	880	F	1	78		0·745		0·4383		58·8		1·43	
			888	F	2	110		0·951		0·5993		63·0		1·70	
			897	M	3	140		1·010		0·6048		59·9		1·49	
			905	M	4	128	114	0·949	0·914	0·5765	0·555	60·7	60·6	1·55	1·54
3 *R*	50 % hog millet	Irradiated cotton-seed oil	898	M	3	141		0·986		0·5930		60·1		1·51	
			906	F	4	100		0·863		0·5315		61·6		1·60	
			912	F	5	103	115	0·961	0·937	0·5898	0·571	61·4	61·0	1·59	1·57
4 *R*	1 % alfalfa meal	Irradiated cotton-seed oil	882	F	1	85		0·801		0·4949		61·8		1·62	
			890	M	2	130		1·023		0·6381		62·4		1·66	
			899	F	3	93		0·896		0·5579		62·3		1·65	
			907	M	4	124	108	1·028	0·937	0·6279	0·580	61·1	61·9	1·57	1·62

SUMMARY.

1. Experiments are described with two diets suitable for the assay of vitamin D, in which vitamin A was provided by 20 % wheat embryo (Hume) or 50 % hog millet (Steenbock, Nelson and Black).

2. Steenbock and Black's observations that alfalfa meal contains too much vitamin D for the purpose are confirmed.

3. The conclusion of Steenbock and his colleagues [1923], that the growth curve of the animal on an otherwise adequate diet can be employed for the assay of vitamin D, is substantiated.

We wish to thank Sir Charles Martin and Dr H. Chick, who instigated this research, for their great assistance and helpful advice, and the Lister Institute for the hospitality of its laboratories. We should also like to thank Dr H. Goldblatt, of Western Reserve University, Cleveland, for kindly supplying the hog millet used in this experiment. Our thanks are also due to the Medical Research Council for a whole time grant.

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LXVII. THE ESTIMATION OF THE DIAMINO-NITROGEN IN THE PRODUCTS OF HYDROLYSIS OF PROTEINS.

By JOSEPH CHARLES KERNOT and JOHN KNAGGS.

From the Fish By-products Research Laboratory, Imperial College of Science and Technology, London, S.W. 7.

(Report to the Food Investigation Board—Department of Scientific and Industrial Research.)

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The discordant results obtained in protein analysis by following the method suggested by Van Slyke [1911, 1915] are attributed by every investigator to factors which arise in the precipitation and treatment of the phosphotungstates of the hexone bases.

Kutscher [1900] and Chittenden and Eustis [1900] state that the precipitation of diamino-acids by phosphotungstic acid is not complete. Osborne, Leavenworth and Brautlecht [1908] in their work on vegetable proteins also noted considerable discrepancies in the results obtained by direct estimation of histidine, arginine, and lysine by the methods suggested by Kossel and Kutscher [1901] and Kossel and Patten [1903], and the results obtained by estimating the same amino-acids by precipitation with phosphotungstic acid. Görtner and Hoffman [1925] found that the temperature at which the precipitation takes place has a considerable influence on the results. This was contradicted by Plimmer and Rosedale [1925] who state that no significant difference was produced by allowing the precipitate to stand at 0° instead of at room temperature. They also state that prolonging the time of standing of the precipitate has no particular advantage. In their opinion the origin of the discrepancies lies in the solubilities of the phosphotungstates.

The experience of the writers in the course of analytical work on a considerable number of proteins has confirmed the views mentioned at first, and the object of the present investigation has been to find out how far the results obtained could be influenced by the temperature at which the precipitation takes place, and by the concentration of the nitrogen in the hydrolysate.

In the course of these investigations ossein and the gelatin obtained from it were the materials used, in addition to dry cod-skins on which we were working at the time for other reasons. The ossein was carefully decalcified, treated for 60 days with 0·2 % NaOH, electrolysed, and treated finally with 0·5 % HCl for 4 days.

The different conditions under which the hydrolysis was carried out are given on the curves Figs. 1 and 2.

In every case, after hydrolysis, the hydrochloric acid, amide-N and humin-N were removed in the usual way, and the residue was taken up with water and filtered.

The solution was made up to a known volume and the nitrogen content determined by Kjeldahl's method.

Aliquot parts of this solution were taken, and the volume was adjusted to 100 cc. The diamino-acids were precipitated from this solution, which contained 5 % H_2SO_4, with an excess of phosphotungstic acid, following the method of Osborne and Harris [1903; see also Drummond, 1918], *i.e.* 30 cc. of phosphotungstic acid were added in each case so that the volume of solution used was constant throughout the experiments.

The basic fraction was precipitated under different conditions of temperature as follows:

(*a*) at 20°, filtered after 24 hours and washed;

(*b*) the solution was heated to 100°, the phosphotungstic acid was added, and the solution was allowed to cool and stand for 24 hours at laboratory temperature, then filtered and washed with a constant volume of liquid.

The mono-amino-N in the filtrate was estimated by the Van Slyke method for amino-N. Figs. 1 and 2 give the results obtained for the percentage of diamino-N.

It is evident from the curves that the following factors play a very important part in the precipitation of diamino-N by phosphotungstic acid.

(*a*) The concentration of the nitrogen in solution.

(*b*) The temperature of the solution during precipitation.

(*c*) The concentration of the acid used for the hydrolysis of the protein.

(*d*) The treatment with cold acid before hydrolysis.

The effect of the last-named factor has been discussed already in a paper by Knaggs [1923]. In the work there described the gelatin was hydrolysed with 20 % HCl but the concentration of nitrogen in solution was not varied, and the precipitation with phosphotungstic acid was carried out at laboratory temperature only.

(*a*) *Concentration of the nitrogen in solution.*

As the concentration of the nitrogen in solution is increased, the percentage of it precipitated by phosphotungstic acid increases also. At high concentrations, especially in the case of gelatin, there is a gradual decrease (Fig. 1, curve A). But this is not the case with ossein, which has been partially hydrolysed in the cold with 20 % HCl, as the amount precipitated increases regularly with the concentration. Ossein and cod-skins give a nearly constant value over a wide range of concentrations.

It is impossible to give a satisfactory explanation of the anomaly shown by gelatin as we have not an exact knowledge of the constituents of its hydrolysis products. The anomaly, however, may be due to influences of solubility.

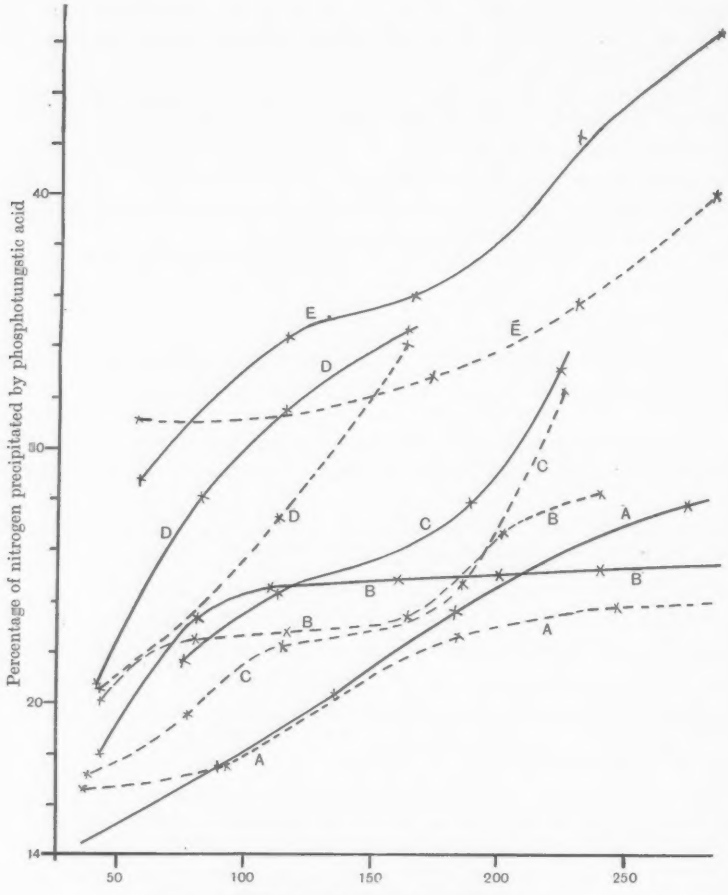

Fig. 1.

A. Gelatin from ossein hydrolysed by 20 % HCl.
B. Ossein hydrolysed by 20 % HCl.
C. Ossein treated with cold 20 % HCl for 10 days and hydrolysed.
D. Ossein hydrolysed by 5 % HCl.
E. Ossein treated with 20 % HCl at 25° for 10 days.

——— Basic nitrogen precipitated from cold solution.
– – – – – „ „ hot „

(b) Temperature of the solution during precipitation.

The percentage of basic nitrogen precipitated from hot solution is generally lower than that precipitated at laboratory temperature. The experiments carried out with cod-skins which had been treated with a cold solution of 0·5 % HCl and then hydrolysed with the same concentration of acid (Fig. 2 D) do not seem to confirm this. The anomaly is probably due to incomplete hydrolysis.

(c) The concentration of the acid used for the hydrolysis.

The percentage of basic nitrogen obtained from the product of hydrolysis with weak acids is higher than that obtained from the precipitation of the diamino-fraction from the hydrolysis products for which more concentrated acids are used. This is shown to be the case for ossein in Fig. 2.

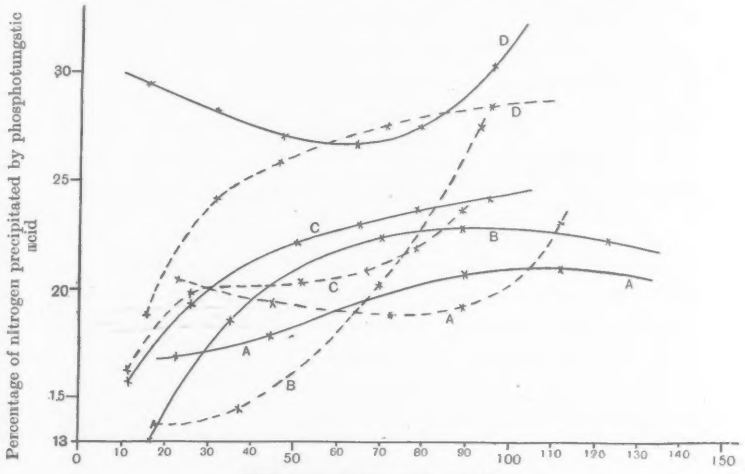

Fig. 2. Dry cod-skins.

A. Immediately hydrolysed with 20 % HCl.
B. Immediately hydrolysed with 5 % HCl.
C. Standing 10 days with 20 % HCl and then hydrolysed at 100° with 20 % HCl.
D. Standing 10 days with 0·5 % HCl and then hydrolysed at 100° with 0·5 % HCl.

– – – – – – Basic nitrogen precipitated from hot solution.
——— „ „ cold „

It is noticed that the basic values obtained from gelatin and ossein are very different. This seems to be evidence in favour of the view expressed by Emmett and Gies [1907] that collagen is not merely an anhydride of gelatin, as suggested by Hofmeister [1878], but an entirely different compound which produces gelatin by an intramolecular rearrangement.

(d) The treatment with cold acid before hydrolysis.

The increase shown in the percentage of basic nitrogen when the protein is allowed to stand with cold acid before hydrolysis is evidence of the fact that other products, probably ring-compounds, are precipitated by phosphotungstic acid. This has been observed in the case of ossein and cod-skins, as shown by Fig. 1 C and Fig. 2 C.

THE RESULTS FOR THE MUSCULAR TISSUE OF FISH.

In the course of investigations on fish muscular tissue, similar results to those given above were obtained. These are given in Table I.

Table I.

Muscular tissue of	N in solution mg.	% of basic N precipitated from cold solution	% of basic N precipitated from hot solution
Shark (red)	40·6	30·9	30·2
	81·2	35·6	31·9
	102·0	42·6	—
Shark (white)	40·8	35·3	32·2
	81·6	42·3	35·4
Cod	84·5	34·4	33·5
	120·0	45·2	—
Haddock	54·3	39·3	33·2
	108·6	44·3	43·1
	142·0	46·4	—

PRECIPITATION OF THE BASES AT 0°.

Experiments were carried out by precipitating the hydrolysate which had been cooled to 0° with phosphotungstic acid solution kept at the same temperature. For comparison aliquot parts of the same solution were precipitated at a temperature of 37°, and in each case the solutions were allowed to stand for 24 hours at the temperature of precipitation before filtration. This was carried out very quickly and the precipitates were washed with dilute phosphotungstic acid which had been kept at the same temperatures. The results are given in Table II.

Table II.

	Nitrogen in 100 cc. solution mg.	% diamino-N precipitated from solution cooled to 0°	% diamino-N precipitated from solution at 37°
Ossein. Allowed to stand 10 days with 5 % HCl and hydrolysed	182·8	37·4	21·7
Cod-skin dry. Hydrolysed with 20 % HCl	112·5	25·7	21·1
Hydrolysed with 5 % HCl	88·5	32·2	19·6
Allowed to stand 10 days with 20 % HCl and hydrolysed	64·9	38·4	23·5
Allowed to stand 10 days with 0·5 % HCl and hydrolysed	79·8	35·6	24·6
Skate-skins. Hydrolysed with 20 % HCl	50·4	20·8	13·9
Hydrolysed with 0·50 % HCl	52·0	24·4	15·5
Allowed to stand with 20 % HCl and hydrolysed	63·0	19·5	17·1

It will be seen from the above results that a considerably greater percentage of diamino-N is precipitated at 0° than at 37°, thus confirming the results of Görtner and Hoffman.

The problem is to establish whether the whole of the precipitate is due to products of a basic nature. This does not seem to be the case, however, according to the above-mentioned authors, who found, amongst the products precipitated, hydroxyaminobutyric acid.

Knaggs [1923] has previously shown how the variations obtained in the Hausmann numbers for gelatin depended on the preliminary treatment of the sample of gelatin or its precursors. In particular the diamino-N showed the greatest variations. It was suggested that the discrepancies were due to the formation of peptides which were resistant to hydrolysis and were precipitated by phosphotungstic acid.

Apart from this possible explanation the suggestion made by Plimmer and Rosedale [1925] that the differences observed may be due to the solubilities of the phosphotungstates in excess of the reagent is worth considering.

In the results shown in this paper it is noticeably the fact that a considerably higher percentage of diamino-N is obtained in all those cases in which the hydrolysis is carried out with dilute acids, thus showing that peptides which are more resistant to hydrolysis are precipitated by phosphotungstic acid. It is evident that there is a great difference in the solubility of these phosphotungstates at the different temperatures, apart from the fact that such phosphotungstates may be hydrolysed at high temperature and therefore will not precipitate on cooling.

Furthermore, it has been shown by different investigators that other hydrolysis products, apart from peptides, are precipitated by phosphotungstic acid. Schulze and Winterstein [1901] showed that certain amino-acids, especially phenylalanine, were precipitated by this reagent.

Görtner and Sandstrom [1925], using artificial mixtures of amino-acids, proved that the presence of proline and tryptophan led to errors in the basic fractions, these substances being partially precipitated.

Admitting that the errors may be due to the causes mentioned by the above investigators, we think that the presence of peptides precipitated by phosphotungstic acid offers a better explanation.

Investigations carried out in this laboratory have led to the isolation of at least two peptides in the diamino-fraction of gelatin from isinglass, one of them soluble in ethyl acetate and identified as alanylalanine (Speer, privately communicated).

The necessity of standardising the technique in the analytical study of the hydrolysis products of proteins by the Van Slyke method becomes more and more evident as the data available increase.

SUMMARY.

The percentages of nitrogen precipitated by phosphotungstic acid from the products of hydrolysis of a protein depend on several factors.

(*a*) The concentration of nitrogen in the solution.

(*b*) The conditions of precipitation, *e.g.* temperature of precipitation and temperature of the solution whilst the precipitate is settling.

(*c*) The concentration of the acid used for the hydrolysis of the protein.

(*d*) Period of contact of the protein with the cold acid before hydrolysis.

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LXVIII. THE VITAMINS OF COMMERCIALLY CONCENTRATED ORANGE JUICE.

By STANLEY GORDON WILLIMOTT.

From the Biochemical Laboratory, Cambridge.

(*Received March 8th, 1928.*)

The problem of conserving the antiscorbutic potency of *Citrus* fruit juices has exercised the mind of investigators since the close of the war. Experimental work along these lines, principally in America and in England, has been carried out by the application of two chief processes, the spray process and the method of concentration *in vacuo*. In 1919, Givens and McClugage pointed out the numerous and obvious advantages that would follow if a satisfactory industrial method could be devised for the concentration of the vitamin C content of fruit juices [1919].

Several different methods of supplying vitamin C for practical purposes have been suggested. Amongst these, mention may be made of the method of germination of pulse [Chick and Delf, 1919; Chick and Hume, 1917, 1, 2; Wiltshire, 1918]; the canning of tomatoes [see Medical Research Council's Report, 1924]; preservation of orange juice, and lemon juice, exposed to ordinary temperatures, by means of the rind oil [Davey, 1921]; concentration of lemon juice *in vacuo*, with the addition of lactose and gum to form potent tablets [Bassett-Smith, 1920, 1921]; and the use of dried orange juice and lemon juice powders (Merrell-Soule). There are, however, different objections to all these methods.

The most satisfactory method so far devised appears to be that of concentration of orange juice *in vacuo* at a low temperature. In this country, Harden and Robison [1919; 1920; Robison, 1919] who appear to have been the first to introduce this method, showed that fruit juices may be dried without serious loss of antiscorbutic potency by evaporation *in vacuo* at 40°. Moreover, the dry residue from orange juice was found to retain some antiscorbutic principle after two years' storage over sulphuric acid at room temperature. On the other hand, Givens and McClugage [1921] employing the spray process found dried orange juice to be still effective after 14–20 months. The spray process of drying orange juice has also been studied in detail by McClendon [1921], who devised a special apparatus. However, the experience of different investigators appears to show that the spray process is inferior to the vacuum process in that it does not produce so satisfactory a product.

The work of Harden and Robison has received ample confirmation. For example, Goss [1925] using concentrated California orange juice (identical

with the product used in the present investigation) found that it retained practically all the original antiscorbutic potency. Hawk, Fishback and Bergheim [1920], employing orange juice evaporated to dryness *in vacuo*, arrived at similar conclusions; while Priston [1926, 1], in an exhaustive study carried out on behalf of the British Navy, substantially confirmed Goss's findings. The question of the preservation of the vitamin C content of different species of orange juice by large scale processes has also been considered by Delf [1921] in her study of some South African foodstuffs.

The problem of the survival of vitamins A and B, known to be present in orange juice [Osborne and Mendel, 1920, 1921; Osborne, Mendel and Wakeman, 1920; Willimott, 1928], has received less attention. However, Osborne and Mendel showed that both these vitamins persisted in undiminished potency when orange juice was desiccated by concentration *in vacuo* in the laboratory.

This important finding, together with the fact that the vitamin C in orange juice is more stable to high temperatures than in other natural antiscorbutic products [Delf, 1920], justified the belief that vitamins A and B could also be retained intact in orange juice which had been commercially concentrated by the vacuum process. To investigate the validity of this supposition the experiments to be described were carried out on a concentrated product prepared from the same species of orange as that previously studied [Willimott, 1927, 1928].

Experimental.

The concentrated orange juice used throughout this work had been prepared in California from fresh ripe navel oranges in the ordinary way of manufacture. The results obtained were therefore strictly comparable with those of the fresh California orange juice (Sunkist brand) previously reported [Willimott, 1927, 1928]. The manufacturers kindly supplied the following information in regard to the product employed. The fresh navel orange juice contained 16·15 % total solids and 1·41 % anhydrous citric acid. The only added material was stated to be a small percentage of pure cane sugar (3·28 %) for the purpose of standardising the sweetness of the product for commercial purposes. The sweetness of orange juice depends upon the ratio of the amount of natural fruit sugar to the amount of citric acid present, which, while naturally varying to some extent, is usually about 12 to 1 in the best quality juice. In the manufacturing process the juice is tested before concentration, and, if necessary, these variations are corrected by adding small but varying amounts of cane sugar, thus standardising the sweetness.

The fruit, selected by hand grading, is first washed, and the juice immediately after extraction is passed into special, glass-enamelled vacuum pans, where it is concentrated at 37°. During the evaporation *in vacuo*, which is carried out very rapidly, the juice comes into contact with no metals. In this way, it is possible to carry the process of concentration to a point, approximately 7 to 1 by volume, where the constituents of the juice are preserved in

its own natural sugars. The resulting concentrate is then drawn off direct from the vacuum pans into carefully sterilised glass jars, which are immediately closed with a sterilised cork and cap.

Product employed. The concentrated orange juice, on arrival in the laboratory, was a thick yellow-brown syrup contained in six sterilised quart glass jars. The specific gravity was 1·3598 and the product gave negative reactions in tests for the oxidising enzymes, while microscopic examination revealed the presence of some *Citrus* tissue. The product was sweet to the taste and slightly acid, with an orange flavour. The sample employed in these experiments was prepared on February 25, 1927, and then, in accordance with the ordinary routine, was immediately placed in cold store and kept under refrigeration conditions until its arrival in London on April 22. It was exposed to ordinary temperatures at the docks for five days (April 22 to April 27), when it was again placed in cold store (1·5 to 4·5°), and remained there until its arrival in Cambridge on May 25. During the course of the experimental work the concentrate was placed in a cold store room, the temperature of which varied from 0° to 1°.

The results of recent analyses made in California, Berlin and Vienna, are summarised in Table I. It will be seen that on the whole the range of variation for the different values is comparatively small.

Table I. *Analyses of concentrated orange juice.*

	California %	Berlin %	Vienna %
Total solids	72·43	72·90	72·48
Invert sugar	30·43	33·80	34·03
Cane sugar	25·79	22·40	23·96
Total acid as citric acid	6·12	6·14	5·76
Remaining extract (by difference)	10·09	10·56	8·73
Matter insoluble in water	—	0·90	0·96
Total soluble solids	—	72·00	71·52
Volatile acids	—	none	none
Ash	1·77	2·02	1·94
Alkalinity of ash as K_2CO_3	1·45	1·57	1·43

Notes on Table I.

All the analyses were carried out on representative samples of the same brand of orange concentrate, drawn at random, at the places stated.

The analyses made in California are the averages of from 33 to 44 separate estimations. The calcium, phosphorus, and potassium present were also determined, and stated to be as follows:

CaO 0·128 %. P_2O_5 0·198 %. K_2O 0·939 %.

In the Berlin analysis, due to Cohn, coal-tar colours and chemical preservatives were not detected.

In the Vienna analysis, carried out at the Staatliche allgemeine Untersuchungsanstalt für Lebensmittel, the polarisation reading (direct, 200 mm.) was given as +15·95°, and −26·87° after inversion. Alcohol, tartaric acid, and sulphurous acid could not be detected. Benzoic acid, salicylic acid, saccharin, and aniline colours were absent.

The experimental work was carried out, as far as possible, under the same conditions and employing the same methods as in the investigation of the fresh California orange juice (Sunkist brand) [Willimott, 1928]. Little, therefore, need be said here. Albino rats from the same stock were employed, and

the methods for assaying vitamins A and B were identical with those then used. The method of administering the concentrate was to take the required amount and dilute it, using distilled water, to the strength of the juice from which it was originally prepared. Samples from each jar were used. The diluted product closely resembled, in appearance and smell, the natural orange juice. The system of dosage was thus on an orange juice basis. The concentrate was found to go into solution in water quite readily with a little shaking. The required doses were then measured out, and fed at once to the experimental animals, each of which occupied a separate cage. The animals received their doses at 10 a.m. in advance of the basal ration. The required amount of prepared orange juice was made up fresh each day, and it was given to the animals in flat-bottomed earthenware pots which could not be upset.

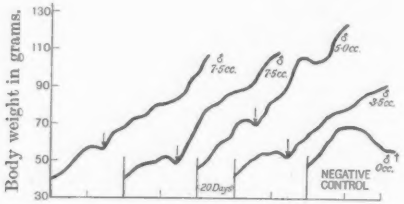

Fig. 1. Vitamin A in concentrated orange juice. (Exploratory groups.)
↓ Concentrated orange juice (diluted to strength of original orange juice) administered.
† Death.

Vitamin A.

Although Goss [1925] had shown that the antiscorbutic factor present in California orange juice could be preserved *in toto* by concentration *in vacuo*, it was not assumed at the outset of the present investigation that the large scale concentration process and subsequent storage had had no effect upon the vitamin A potency of the original juice. An exploratory group was therefore set up, which consisted of five male albino rats, and placed upon the vitamin A-deficient diet usual to this laboratory. Vitamin D was supplied by means of irradiated cholesterol suspended in liquid paraffin. When growth had ceased the following doses were administered. Two animals received 7·5 cc. of diluted orange concentrate, a third animal 5·0 cc., a fourth 3·5 cc., and a negative control received no orange juice. The weight changes resulting from the ingestion of these doses are illustrated in Fig. 1. These preliminary results were considered to indicate that a dose of 5 cc. of the diluted orange concentrate was probably adequate in vitamin A for the requirements of the albino rat.

A large confirmatory group, consisting of five male and six female animals, was next run out on the vitamin A-deficient diet, vitamin D being supplied

as before. Growth had either ceased or was declining about the fifth week when incipient xerophthalmia began to appear. At the point where dosage commenced, the food intake had fallen from 8 g. to about 6 g. per head per day. One animal, perhaps the weakest of the group, receiving three drops daily of a good cod-liver oil (as indicated by colour test), acted as positive control. The animal which appeared to be the strongest of the group was employed as the negative control. This animal (female) died on the 51st day of experiment; symptoms of vitamin A-deficiency were found at autopsy. The remaining nine

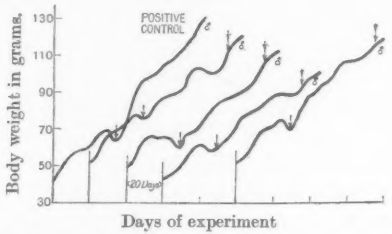

Fig. 2 *a*. Vitamin A in concentrated orange juice (male group).

↓ Orange concentrate, diluted to the strength of the juice from which it was originally prepared, administered as 5 cc. dose.

↓ 10 cc. administered.

↓

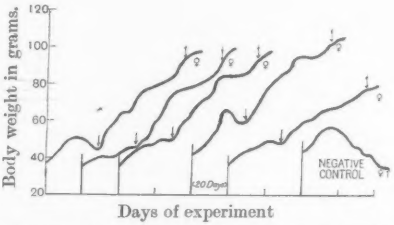

Fig. 2 *b*. Vitamin A in concentrated orange juice (female group).

↓ Orange concentrate, diluted to the strength of the juice from which it was originally prepared, administered as 5 cc. dose.

↓ 10 cc. administered.

↓

† Death.

animals each received a dose of 5 cc. of diluted orange concentrate per day, and this was readily consumed in every case. All the rats receiving orange concentrate responded quickly, and on the whole uniformly good growth was manifested throughout the six weeks during which this dose was administered. Beyond the fact that the growth of these rats was perhaps a little less regular, there was little difference between these and the positive control receiving an adequate amount of vitamin A from a known source.

On the 75th day of experiment, the dose was raised to 10 cc. per head per day (see Figs. 2 *a* and 2 *b*). In the majority of cases this increased dose was

also consumed completely, but its ingestion did not appear to have any marked effect upon the growth rate during the short period it was fed. Onwards from the time when dosage first commenced, the orange concentrate supplied vitamin C as well as the necessary vitamin A. The group was therefore deriving its requirement of these two vitamins from this product during a period of about eight weeks. At the conclusion of the experiment, all the surviving animals appeared quite normal in every way. At autopsy, except for indications of some lung congestion, normal conditions were found, while the state of nutrition was indicated by the presence of normal subcutaneous fat. The food intake averaged approximately 9 g. per day.

Vitamin B.

As in the feeding experiments on fresh California orange juice, the vitamin B content (composite factors) of the concentrated product was tested on rats, but the antineuritic value was not estimated. The vitamin B-deficient diet was identical with that previously employed [Willimott, 1928], *i.e.* lemon juice was omitted and potato starch was replaced by rice starch. From the experience previously obtained with fresh orange juice, and the fact of the stability of vitamin B, preliminary experiments in this case appeared to be unnecessary. Accordingly a large group, consisting of four male and eight female rats, whose initial weight averaged about 50 g. each, was placed on the vitamin B-deficient diet. The animals were now placed in separate cages of a mesh wide enough to allow the faeces to fall clear of the cage. Failure occurred between the 14th and the 20th days, as evidenced by loss of weight, lack of appetite, staring coats, and in some cases by symptoms of paresis. The food intake at this point was less than 4·5 g. per head per day. A dose of 10 cc. of diluted orange concentrate was then given to each of ten animals before the basal ration was administered each day. The remaining animals acted as positive and negative controls respectively (see Figs. 3 *a* and 3 *b*).

As a result of work on the vitamin B content of yeast extracts, which it is hoped to publish elsewhere, 0·75 cc. of an extract of marmite appeared to be about the minimum adequate dose per day for the vitamin B requirement of the albino rat. This dose was fed to the positive control (male), which responded promptly: on the average, the animal gained about 13 g. per week. The negative control (female) died on the 29th day of experiment, the appetite having failed almost completely sometime before death supervened. The symptoms at autopsy, which were not very definite, indicated death from inanition.

Because of the marked failure of appetite characteristic of this deficiency, the problem of ensuring complete dosage was more difficult than in the case of the feeding experiments for vitamin A. However, the dose of 10 cc. of diluted orange concentrate, after the first week of its administration during which the animals had become accustomed to it, was taken completely by all except three animals throughout the eight weeks of dosage. As was observed in the feeding experiments with the fresh orange juice, there were similar

variations in the amount of the 10 cc. dose actually consumed by the remaining three animals.

Efforts were not spared to induce these animals, if possible, to consume the specified dose *in toto*, and to this end, the dose was given in the form of undiluted concentrate, at half the strength of normal orange juice, and lastly at full strength but sweetened by the addition of a little cane sugar. However, after trying these different methods, the procedure of giving the specified dose at the strength of normal orange juice was adhered to, as it was found to be the most satisfactory. The amount of diluted orange concentrate not con-

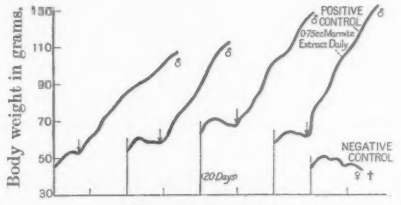

Fig. 3 *a*. Vitamin B in concentrated orange juice (male group).

↓ Orange concentrate, diluted to the strength of the juice from which it was originally prepared, administered as 10 cc. dose.

† Death.

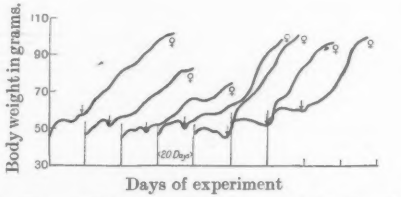

Fig. 3 *b*. Vitamin B in concentrated orange juice (female group).

↓ Orange concentrate, diluted to the strength of the juice from which it was originally prepared, administered as 10 cc. dose.

sumed by these three animals was small and varied daily from 0·5 to 2 cc. Moreover, it did not appear to have any definite effect upon the growth of the animals concerned. After receiving the orange concentrate the appetite improved, for it was observed that the food intake rose steadily up to 8 g. per head per day. At the conclusion of the experiment the group was in normal health and in a satisfactory state of nutrition. Careful autopsies were carried out on a number of animals including the control, but no pathological conditions were found. It was concluded therefore that the dose of 10 cc. of diluted orange concentrate was adequate for the supply of the vitamin B requirement of the animals.

Discussion.

In a previous investigation [Willimott, 1928] it was shown that 5 cc. and 10 cc. of fresh ripe California Sunkist orange juice were doses adequate in vitamin A and vitamin B respectively for the requirements of the albino rat. Using the same breed of animals and an identical experimental technique, a product prepared from the juice of the same species of orange by concentration *in vacuo* was found to meet the nutritive needs of the animals as regards vitamin A and vitamin B at the same level in each case. The two investigations were run in close succession and the conditions of experiment were maintained as far as possible identical.

The experimental findings can therefore be compared with some degree of confidence. From this it can be concluded that the content of vitamin A and vitamin B of fresh ripe California orange juice survives the industrial process of concentration by the method of vacuum distillation at 37° without apparent loss, at least in the case when the resulting concentrate is of fairly recent manufacture.

The antiscorbutic potency of fresh California orange juice has been carefully investigated by Goss [1925], who showed that the minimum daily dose for the guinea-pig was 1·5 cc. of the fresh ripe juice. This investigation was extended to embrace different *Citrus* concentrates. It was found by Goss, in the case of the same orange concentrate prepared from California oranges, as used in the present investigation, that the content of vitamin C was practically unaltered. These findings have received independent confirmation in the work of Priston in this country on behalf of the British Navy [1926, 1, 2].

It thus appears definite that, so far as our knowledge extends, the three vitamins (A, B and C), known to be present in fresh California orange juice in appreciable amount, are preserved apparently without loss when concentrated by a carefully controlled industrial process of distillation *in vacuo* at low temperature.

Such a concentrate, prepared under these conditions, is sterile, owing to the high osmotic pressure of the sugars present, and is of high bactericidal power [Priston, 1926, 1, 2]. It also appears to keep well over long periods [Priston, 1926, 1; Humphrey, 1926]. However, when the concentrated product is diluted to the strength of raw orange juice it ferments on standing for a few days, and moulds and bacteria can then be readily grown. Because of its sugar content, the energy value of the concentrate appears to be high. The slightly bitter taste sometimes observed in such concentrates is probably due to the presence of the bitter glucosides sometimes found in the orange. These constituents appear worthy of further study, especially as their presence in concentrates may affect the flavour. The pigments of the orange are also deserving of further investigation [cf. Palmer, 1922]. Other important constituents are the basic mineral matter present in orange juice, which is retained intact in concentrates derived from it [see Chaney and Blunt, 1925], and the protein [Smith, 1925].

The experimental results of feeding tests carried out by different workers on fresh California orange juice and on the concentrated product derived from it, are summarised in Table II.

Table II. *The vitamin content of fresh and concentrated California orange juice.*

	Minimum adequate dose			
	Fresh juice cc.	Concentrated juice (diluted to strength of fresh juice) cc.	Animal	Observer
Vitamin A	5	5	Rat	Willimott [1928]
Vitamin B	10	10	Rat	Willimott [1928]
Vitamin C	1·5	1·5	Guinea-pig	Goss [1925]
Vitamin D	Not found	—	Rat	Willimott [1928]
Vitamin E	Variable	—	Rat	Evans and Burr [1927]

SUMMARY.

(1) Concentrated orange juice of known origin, history and manufacture was used throughout this investigation.

(2) Independent analyses of this product, made recently at different centres, are embodied in a table.

(3) Working under the same conditions as in the fresh orange juice enquiry, it was found that 5 cc. of orange concentrate, diluted to the strength of the original juice, was adequate in vitamin A for the rat.

(4) 10 cc. of the diluted orange concentrate was adequate in vitamin B.

I should like to express my thanks to Sir F. G. Hopkins for his interest in this investigation and to the California Fruit Growers' Exchange, Products Department, who kindly provided the material employed.

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LXIX. DETERMINATION OF UREA IN BLOOD.

By FRANCIS WILLIAM FOX.

From the South African Institute for Medical Research, Johannesburg.

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THE methods for the determination of urea in blood described below have been developed from a procedure described by Kennaway [1920]. The ammonia produced by the action of urease is determined directly and without previous aeration; consequently no special apparatus is required and the possible error due to incomplete removal or loss of ammonia is avoided.

The macro-method has proved most reliable and simple as a routine procedure.

MACRO-ESTIMATION OF UREA IN BLOOD.

Solutions required (1) 10 % sodium tungstate; (2) 0·75 *N* H_2SO_4; (3) urease extract; made by shaking about 1 g. of powdered soya bean with 50 cc. of 0·85 % saline and filtering after 20 minutes.

Method. Measure 5 cc. of blood into a 175 cc. conical flask, followed by 5 cc. of 0·75 *N* H_2SO_4 and 5 cc. of 10 % sodium tungstate.

Now add 35 cc. water and shake thoroughly. Filter into a 50 cc. measuring cylinder. Place 30 cc. of filtrate into another 175 cc. conical flask and wash out the cylinder with 10 cc. water. Add 3 drops 0·2 % methyl red or chlorophenol red, boil and cool under the tap. With each batch of estimations a blank is run in order to allow for the alkalinity of the enzyme extract. This consists of 40 cc. of water placed in a similar flask with an equal quantity of indicator. Adjust to a reddish yellow colour by means of *N*/100 acid or alkali. Now neutralise the test, first approximately by means of *N*/10 alkali and finally match with the blank by means of *N*/100 alkali. Add 5 cc. of enzyme extract to both blank and test. Incubate for 1 hour at about 50°. Add *N*/100 acid in 10 cc. lots until the solution is definitely acid. Most samples will not require more than 10 cc. The blank receives 10 cc. Boil, cool under the tap and titrate with *N*/100 alkali matching the test against the blank. The volume of alkali run in is subtracted from that required for the blank and the difference multiplied by 10 gives the percentage of urea in the blood.

The sensitivity of the method depends largely upon the sharpness of the end-point. Even with *N*/100 alkali in the presence of blood-salts and bean extract this is surprisingly sharp, provided (*a*) that the amount of indicator

added is not too great and is the same as that used for the blank, (*b*) that the minimum quantity of potassium oxalate is used as anticoagulant; 1–2 drops of a 20 % solution are ample for 10 cc. blood.

If these conditions are carefully observed a few drops of the alkali produce an unmistakable change in shade.

Micro-estimation of urea in blood.

A 4 in. × ½ in. test-tube is calibrated to hold 10 cc. Place 8–9 cc. water in the tube and then run in 0·25 cc. of blood, washing the pipette out with the water. Now add 0·25 cc. 0·75 *N* H_2SO_4, and, after mixing, 0·25 cc. 10 % sodium tungstate. Mix again. Wash down the sides with a little water bringing volume almost up to 10 cc. Centrifuge for 5–10 minutes at about 2000 revs. The supernatant fluid must be water-clear. Make up to the 10 cc. mark and drain off into an accurate 10 cc. measure. Read volume—about 8–9 cc.—and transfer to a 50 cc. conical flask washing out the measure with 2 cc. water. Add 1 drop of 0·2 % methyl red from a capillary pipette. Boil, cool. Neutralise, first roughly with *N*/10 NaOH and finally with *N*/500 alkali or acid. Two blanks consisting of 12 cc. water and a drop of indicator are set up and brought to neutrality. The test is matched against them. Now add 0·5 cc. urease solution to each flask and incubate at 50° for ½ hour. After incubation run in *N*/500 acid from a micro-burette until the solution gives a marked red colour. Note the volume. The blanks should each receive 1 cc. of acid. Now boil, cool and titrate with *N*/500 alkali. The number of cc. of acid neutralised by the ammonia multiplied by 300 and divided by the volume of supernatant fluid used represents the urea in mg. %.

Notes. (1) The blood will usually be taken from a finger and delivered direct into the test-tube. If this is not convenient it is absolutely essential that the minimum quantity of oxalate is used.

(2) It is important to use the same amount of indicator in both the test and the blank. If this is done there should be no difficulty with the end-point, at any rate in good daylight. The two blanks are placed on either side of the test during titration and take the place of a comparator.

Table I. *Comparison of macro- and micro-methods.*

	Urea mg. %																		
Macro-method (5 cc. oxalated blood)	68	274	89	30	57	23	116	34	211	35	123	44	37	47	48	59	45	38	24
	43	13	26	224	69	26	47	33	63	39	49	60	42	65	27	41	173	48	51
Micro-method (0·2 cc. oxalated blood)	73	256	82	32	53	19	109	30	188	36	134	46	37	43	39	57	36	33	23
	39	20	29	247	68	27	47	37	62	39	53	58	44	59	25	42	180	50	54

We do not claim for this method the accuracy obtainable with some of the more elaborate procedures available (see review by Klisiecki [1926]), but suggest that it has the advantage of simplicity. In our hands it has given results which agree fairly well with those obtained on the larger quantities of blood. These comparisons were made without undue attention to detail so

that an opinion could be formed of the order of accuracy that might be expected under routine conditions.

In Table II this method was used to follow the rise in the blood-urea after administration of 15 g. urea.

Table II.

		Subject		
		A.	B.	C.
Blood-urea mg. %	Initial	38	30	36
	½ hour	54	43	54
	1 hour	—	52	55
	1½ hours	76	52	55
	2 hours	77	—	50
	2½ hours	51	51	50

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LXX. STUDIES IN CALCIUM METABOLISM.

II. THE CALCIUM CONTENT OF CORPUSCLES, PLASMA, AND SERUM.

By CORBET PAGE STEWART and
GEORGE HECTOR PERCIVAL (*Grocers' Company's Research Scholar*).

From the Departments of Medical Chemistry, Pharmacology and Therapeutics, University of Edinburgh.

(*Received January 31st, 1928.*)

Introduction.

Attention has been directed to the presence of calcium in the serum in colloid combination and in crystalloid form. There is no direct information, however, as to whether the amount of calcium in the plasma is equal to that of the serum or whether it exists in similar forms in the two fluids. Further, there is some divergence of opinion as to the mere presence of calcium in the corpuscles, and in this connection the findings of different workers are by no means concordant. It is on account of the paucity of data and the lack of unanimity of opinion on these points that the present research has been carried out. The investigation includes quantitative analyses of the calcium in true plasma, citrated plasma, serum, and corpuscles, and a study of the physical state of the calcium in serum and in plasma.

The calcium content of plasma, citrated plasma, and serum.

Experimental methods. Cats, anaesthetised by paraldehyde and ether, were used as the experimental animals. Coagulation of blood was delayed and true plasma obtained in the following manner. Blood was withdrawn from the carotid artery through a paraffined needle into a paraffined glass syringe, and transferred to ice-cold centrifuge tubes under paraffin. The blood was then centrifuged at high speed for 3 minutes, the centrifuge tubes being surrounded by freezing mixture. 2 cc. samples of plasma were then immediately transferred to centrifuge tubes and oxalated for calcium estimation. To the remaining plasma sufficient sodium citrate was added to obtain a 1·5 % concentration (except in sample 3 *A*, where 0·6 % concentration was used), the mixture thoroughly shaken and 2 cc. samples were taken for analysis. The remainder of the citrated plasma was then centrifuged at high speed for 3 minutes and the calcium content estimated. In some cases, whole blood was citrated directly, centrifuged, and the citrated plasma analysed, and here it was found

that a concentration (0·4 %), roughly corresponding to 0·6 % in serum, was required to prevent coagulation. In all cases a sample of blood was allowed to clot in order to obtain serum therefrom. Kramer and Tisdall's [1921] method of calcium estimation was used throughout except that, following the authors' usual practice, 24 hours were allowed for precipitation of calcium oxalate. In most animals two samples of blood, withdrawn at intervals of about an hour (marked *A* and *B*, in Table I) were studied. The results are set forth in Table I, and include one set of similar observations on a human subject. Table II gives the figures for the calcium content of cat's serum after the addition of increasing amounts of sodium citrate.

Table I.

		Mg. calcium per 100 cc.			
Sample	True plasma	Citrated plasma	Citrated centrifuged plasma	Plasma from citrated blood	Serum
1	12·10	12·00	10·80	—	10·35
2	11·80	11·8	10·66	—	10·50
3 *A*	11·70*	11·60	11·70	—	10·35
B	11·80	11·70	10·20	—	10·35
4 *A*	9·95	10·05	8·85	—	9·00
B	10·00	10·00	9·00	—	9·00
5 *A*	12·1	—	—	10·00	10·0
B	12·05	—	—	10·05	10·0
6	11·10	—	—	10·05	10·0
7 *A*	12·15	—	—	9·50	10·10
B	12·10	—	—	9·50	10·05
8 *A*	11·05	—	—	9·15	9·0
B	11·05	—	—	9·15	9·0
9	12·70†	—	—	11·50	11·30

* 0·6 % sodium citrate added to plasma. † Human blood.

Table II. *Effect of sodium citrate on the calcium content of cat's serum.*

Sodium citrate in serum %	Mg. calcium per 100 cc. serum		
	I	II	III
0·00	10·0	10·35	10·05
0·5	10·0	10·35	10·00
1·0	9·9	10·1	10·00
2·0	9·1	8·0	9·05

The figures recorded in Table I clearly show that the calcium content of true plasma is from 10–20 % greater than that of the corresponding serum. The addition of sodium citrate to true plasma in a concentration of 1 % causes a certain amount of calcium to be precipitated. The evidence for this statement is that if such citrated plasma is centrifuged, the calcium content is reduced, while the calcium content of samples withdrawn for analysis before centrifuging is approximately equal to that of the true uncitrated plasma. The calcium content of citrated centrifuged plasma is roughly equal to that of the corresponding serum. Table II shows that the addition of sodium citrate to serum, in a concentration equal to that just necessary to prevent

coagulation in blood, does not alter its calcium content; on the other hand, when a 2 % concentration has been reached a definite reduction in the calcium content, due to precipitation, is brought about. These observations on serum explain the figures recorded in Table I, sample 3, *A* and *B*. In *A* the concentration of sodium citrate in the plasma is only 0·6 %, an amount which did not cause precipitation in the serum experiments, and no reduction in the calcium content of the citrated centrifuged plasma has occurred. In *B* a 1·5 % concentration of citrate has been used with the result that the calcium content of the citrated centrifuged plasma is lower than that of the true plasma and equal to that of the serum. Kramer and Tisdall [1922] state that they found the calcium content of citrated plasma to be practically the same as that of serum, and they quote Schmidt [1850] as having shown the inorganic composition of plasma to be identical with that of serum. Howland and Kramer [1921] and Halverson, Mohler and Bergeim [1917] could find no appreciable difference between the calcium content of serum and that of plasma. It may be pointed out that these workers give the normal serum value as 9–11 mg. calcium per 100 cc. serum, usually below 10·5 mg., a range which is wider than that found by the present authors [Percival and Stewart, 1926] but is of the same order. They do not state whether they examined true or citrated plasma. It has been pointed out by Kramer, Tisdall and Howland [1921] that the addition of citrate to blood may result in the precipitation of part of the calcium as phosphate, and that the amount of fluid in which the precipitation is accomplished and the length of time allowed for precipitation are important factors. Cameron and Moorhouse [1925], while stating that the calcium values in citrated plasma and in serum are usually equal, record the fact that in two of their experiments the citrated plasma values were definitely higher than those of the serum. The work described in the above and similar papers appears to have been done with plasma prevented from clotting by the addition of citrate or some other anti-coagulant and is therefore not comparable with the work described in this paper, in which true plasma was used.

It may therefore be concluded from the present work that the calcium content of plasma is greater than that of serum, and that the calcium content of citrated plasma is approximately equal to that of serum, although the closeness of this approximation depends entirely on the amount of citrate used to prevent coagulation, and their complete agreement is purely a matter of coincidence.

Calcium content of corpuscles.

The findings recorded in Table III are in agreement with those of Kramer and Tisdall [1922] who concluded, from estimations of the calcium content of whole blood and serum, in conjunction with haematocrit readings, that "the corpuscles of human blood do not contain appreciable amounts of calcium." In their paper they discuss the results obtained by previous workers and point out that in those instances where the presence of calcium

in the corpuscles has been reported, faulty methods of analysis have been employed. More recently Rona, Petow and Wittkower [1924] have confirmed Kramer and Tisdall's results.

On the other hand, Stanford and Wheatley [1925] claim that human corpuscles contain from 1·4–3 mg. calcium per 100 cc. These authors determined the calcium in corpuscles, whole blood and plasma (using lithium citrate as anti-coagulant) by direct analysis, and state that, though the corpuscles doubtless contained adherent plasma, a 20 % contamination would be necessary to explain the results obtained.

In our own experiments, corpuscles were obtained either by rapidly centrifuging blood drawn under paraffin into a vessel surrounded by a freezing mixture, and pouring off the uncoagulated plasma, or by centrifuging defibrinated blood.

By either method the corpuscles gave the same result when submitted to analysis. To remove adherent plasma, the corpuscles were twice washed with 0·9 % NaCl, from which they were separated by centrifuging. The washings contained very small amounts of calcium, no greater than would be expected from the adherent plasma.

Table III.

	Mg. Ca in corpuscles from 100 cc. blood
I	0·30
II	0·20
III	0·15

Distribution of calcium in plasma and serum.

Having determined that the calcium content of the plasma is greater than that of the serum, it was thought advisable to ascertain, if possible, whether a similar difference in concentration exists between the diffusible and ionic fractions of the plasma-calcium and the corresponding serum fractions. Before describing the experiments made for this purpose it is desirable to survey briefly the available methods of estimating the diffusible and ionic calcium in the serum, and also the results obtained by these methods.

So far as the ionic calcium is concerned, the only methods on which any reliance can be placed are those of Neuhausen and Marshall [1922] and Brinkman and Van Dam [1920], which, as reported by those authors, have yielded roughly concordant results. The present authors have carried out a considerable number of determinations of $Ca^{\cdot\cdot}$ using the method of Brinkman and Van Dam, and have obtained figures (Table IV) of the same order of magnitude as those published by those authors and by Neuhausen and Marshall.

Some 80 % of the serum-calcium, then, is present in combination, part doubtless with simple inorganic radicles such as phosphate and bicarbonate, and part possibly with protein. Thus one would expect to find combined calcium partly among the crystalloids and partly among the colloids, in which

Table IV. *Concentration of calcium ions in blood-serum; determined by the method of Brinkman and Van Dam.*

		Mg. $Ca^{..}$ per 100 cc.	
		Whole serum	Serum filtrate
Ox serum	I	2·26	2·26
	II	2·04	2·10
	III	2·54	2·40
Cat serum	I	—	1·90
	II	1·80	1·90
Human serum	I	2·26	2·18

case dialysis should effect a partial separation. The separation need not be complete, however, for disturbance of the equilibrium, it is to be expected, would be followed by a re-arrangement of the relative amounts of dialysable and non-dialysable calcium. Such a method of simple dialysis (which has been proposed by Moritz [1925], Updegraff, Greenberg and Clark [1926] and others) can give results which may approximate to the true one if the time required be short and the rate of readjustment of the equilibrium within the membrane be small; they cannot, however, give the true result. Similar objections may be raised against the method of compensatory dialysis as proposed by von Meysenberg, Pappenheimer, Zucker and Murray [1921] and others, for compensation, to be complete, must take account not only of calcium, but of all the other diffusible substances as well. However, both types of dialysis agree in showing that 50–60 % only of the total calcium is capable of passing through the semi-permeable membrane, and this figure may be accepted as indicating at any rate the order of magnitude of the amount of calcium in crystalloid form of combination.

Very similar results are obtained simply by filtering serum through a collodion membrane under pressure. Cushny [1920] found that only about 60 % of the calcium was filterable in this way, while the whole of the sodium and chlorine passed through the membrane. The pressure under which the filtration is carried out, however, exerts a considerable influence on the result obtained. Cushny used a pressure of 150 mm. of mercury; Richter-Quittner [1921] using very low pressures, found that only 5–7 % of the calcium filtered. Very high pressures, of course, are objectionable, for apart from the technical difficulty of obtaining a protein-free filtrate when they are employed, they tend to destroy any labile compounds between the protein and inorganic ions.

Cushny's figures for the collodion-filterable calcium are in good agreement with those reported by other workers using dialysis, and there can be no doubt that with proper precautions both types of method give comparable results. In measuring the diffusible calcium the present authors have always used the collodion filtration method as being reasonably rapid and sufficiently easily standardised. The collodion membrane must be capable of giving a protein-free filtrate (as determined by the biuret test) but must allow of such a rate of filtration at a pressure of 150 mm. that enough filtrate for analysis can be

obtained in 2 or 3 hours. In a previous paper Percival and Stewart [1927] have shown that the amount of filterable calcium is appreciably increased if the time of filtration is unduly prolonged, and have indicated the method of filtration which they have adopted. Briefly they wash out the envelope with the serum to be filtered, dry the outside with filter-paper, discard the filtrate of the first half hour, and again dry the outside of the envelope before collecting the sample for analysis. The constancy of the results obtained in this way, together with the lack of effect on the results of exposure to air, is shown in Table V.

Table V.

A. Membrane surface dried by filter-paper (Serum LXI).

Time of filtration (hours)	Mg. Ca per 100 cc. filtrate
0–3	4·4
3–7	7·4
7–22	8·5

B. Membrane under pressure 1 hour before commencement of filtration and then dried (Serum LXI).

0–1·5	7·45
1·5–7	7·60
7–24	8·70

C. Treatment as in text (Serum LV).

$\frac{1}{2}$–2$\frac{1}{2}$	7·30
$\frac{1}{2}$–2$\frac{1}{2}$	7·40
$\frac{1}{2}$–2$\frac{1}{2}$	7·35

D. Treatment as described in text, but blood drawn, allowed to clot, and filtered under paraffin (Serum LV).

$\frac{1}{2}$–2$\frac{1}{2}$	7·25
$\frac{1}{2}$–2$\frac{1}{2}$	7·35

Vines [1921] described a biological method for the determination of calcium. This method consisted in comparing the power of the fluid under investigation with that of known concentrations of calcium chloride to restore coagulability to oxalated blood. By its use he found that, whereas uncoagulated blood contains about 6 mg. of calcium per 100 cc., serum contains 10 mg. per 100 cc. and he concluded that the difference represented un-ionised calcium which was not determined by his method—*i.e.* that in blood only 60 %, but in serum 100 %, of the calcium is ionised. Now it is obvious that this conclusion is only justified if it be shown that plasma and corpuscles contain equal concentrations of calcium. Since it is now known that corpuscles contain practically no calcium, and since the plasma forms about 60 % of the total blood volume, it is evident that what Vines really did was simply to determine the total calcium in each case.

In his monograph on *The parathyroid glands in relation to disease* Vines [1924] states that about 60 % of the total *plasma*-calcium is readily precipitated by its chemical equivalent of ammonium oxalate, whereas the remaining 40 % requires a considerable excess. He terms this readily precipitable fraction the "active calcium" of the plasma, abandoning, apparently, the idea that it is ionic. In normal serum, he states, all the calcium is readily

Table VI. *Effect of varying concentrations of oxalate on precipitation of serum-calcium in 24 hours.*

Mg. calcium per 100 cc. equivalent to oxalate added	Mg. calcium per 100 cc. precipitated in 24 hours		
	I	II	III
0·00	0·00	0·00	0·00
2·4	1·30	1·20	1·50
4·8	3·20	1·50	2·60
7·5	5·50	3·55	3·95
9·9	6·40	4·28	5·10
12·5	6·80	4·45	5·85
15·0	7·60	5·25	6·40
19·8	7·60	6·70	7·00
30·0	7·80	—	7·50
∞	9·20	10·55	10·10
Diffusible calcium mg. per 100 cc.	6·20	5·30	5·00

precipitable, though in certain pathological cases this is not so. The statement that calcium is differently distributed in plasma and in serum is so important that it was necessary to subject it to further experiment. Three series of experiments were therefore carried out. In the first series ox serum was treated as for the Kramer-Tisdall determination of calcium, except that measured volumes of a $N/50$ solution of sodium oxalate were used. Precipitation was allowed to continue for 24 hours, after which the calcium oxalate was centrifuged off and determined in the usual way. The results (Table VI) showed that the amount precipitated was a function of the oxalate added, but that in no case did the oxalate precipitate its chemical equivalent of calcium. Further, the amount precipitated by oxalate equivalent to the total calcium of the serum, far from being 100 %, varied from 40–60 % of the total. The second series of experiments (Table VII) showed that the amount of calcium precipitated from serum by small amounts of oxalate depended on the time allowed for precipitation—a fact which has been shown to hold even when a huge excess of oxalate is added [Pincussen and Schimmelpfeng, 1927]. In all cases, precipitation appears to be complete after 24 hours. The third series of experiments was designed to show whether or not the calcium precipitated

Table VII. *Effect of time on the precipitation of serum-calcium by small amounts of oxalate.*

Time allowed for precipitation	Mg. calcium precipitated from 100 cc. serum	
	Oxalate equivalent to 10 mg. calcium per 100 cc.	Oxalate equivalent to 20 mg. calcium per 100 cc.
5 mins.	2·10	3·20
30 ,,	4·65	7·05
1 hour	4·80	7·60
1½ hours	5·12	8·02
2½ ,,	5·20	8·22
3 ,,	5·40	8·35
4 ,,	5·65	8·50
5 ,,	5·82	8·75
24 ,,	5·95	9·20

by small amounts of oxalate was entirely diffusible—that it might be, but was not necessarily, quantitatively equal to the diffusible calcium was shown in the experiments of the first series. To 50 cc. of serum, therefore, oxalate was added in amount approximately equivalent to the total calcium (actually to 10 mg. Ca per 100 cc.) and the mixture was allowed to stand 24 hours, until precipitation was complete. It was then centrifuged, and the total and diffusible calcium were determined in the supernatant fluid. The total and diffusible calcium in the original serum having been determined, subtraction showed (Table VIII) that the calcium precipitated by the equivalent of oxalate was derived from both the diffusible and non-diffusible calcium of the serum, though chiefly from the former. It is evident then that the amount of calcium precipitated by oxalate is a function both of the amount of oxalate and the time allowed for precipitation, and that, even when these conditions are standardised, any agreement with the amount of diffusible calcium is purely fortuitous. Further, it is evident that the whole of the serum-calcium is not "readily precipitable" in the sense that it is precipitated by its chemical equivalent of oxalate.

Table VIII. *Distribution of calcium in serum (mg. %) after partial precipitation by oxalate.*

		I	II
Total calcium in serum		9·20	9·80
Diffusible calcium in serum		6·20	5·70
Total calcium precipitated in 24 hours	by sodium oxalate	6·40	6·70
Diffusible calcium precipitated in 24 hours	equivalent to 10 mg.	5·00	4·50
Non-diffusible calcium precipitated in 24 hours	Ca per 100 cc.	1·20	2·20

From the foregoing statements it is obvious that any method of estimating the amount of ionic or diffusible calcium in serum is entirely unsuitable for similar estimations in plasma on account of the time necessary for satisfactory precipitation, filtration, or dialysis to take place, unless some anti-coagulant substance is added to the plasma. A subsequent paper shows that anti-coagulants such as citrates, fluorides, oxalates, and sodium chloride cannot be employed since they alter the ionic content and disturb the normal ratio of diffusible to non-diffusible calcium in the serum, when added in sufficient concentration to prevent the coagulation of plasma. Under these circumstances it was decided to investigate the possibility of estimating the ionic and diffusible fractions of the plasma-calcium by utilising the peritoneum of the cat as a semi-permeable membrane, carrying out the diffusion *in vivo* in the anaesthetised animal and comparing the findings with those in the ultra-filtrate of the corresponding serum.

The following is a description of the methods employed in these vivi-diffusion experiments. The cat having been anaesthetised with paraldehyde and ether, a long mid-line incision was made through the abdominal wall, extending down to, but not including, the peritoneum; all bleeding points were secured, and oozing was stopped by cauterisation. A small opening was made

into the peritoneal cavity, a measured quantity of either calcium-free, or calcium-containing Ringer solution was introduced, and finally the peritoneal incision was closed by means of a clip. Diffusion between this fluid in the peritoneal cavity and the circulating blood was allowed to proceed for 2–6 hours, samples of fluid being withdrawn for analysis at varying intervals. Samples of blood, for serum, were collected during the course of the experiment. The results of these experiments are recorded in Tables IX and X.

Table IX.

Exp.	Time in hours	Mg. Ca per 100 cc. serum			Fluid introduced into peritoneal cavity	Amount recovered cc.	Mg. Ca per 100 cc. fluid peritoneal cavity		
		Total	Diffusible	Ionic			Total	Diffusible	Ionic
I	0	—	—	—	100 cc. Ca-containing	—	8	—	—
	5	9·00	5·05	—	Ringer solution	25	5·35	5·35	—
II	0	—	—	—	100 cc. Ca-containing	—	8	—	—
	5	9·70	6·55	—	Ringer solution	20	7·00	7·00	—
III	0	11·1	—	—	100 cc. Ca-containing	—	5·65	—	—
	2	11·3	8·1	—	Ringer solution	60	8·0	—	—
IV	0	11·6	8·7	—	100 cc. Ca-containing	—	5·65	—	—
	2	—	—	—	Ringer solution	62	8·8	8·7	—
V	0	—	—	—	100 cc. Ca-free	—	0	0	0
	5	9·25	6·20	1·8	Ringer solution	15	6·5	6·1	1·9
VI	0	—	—	—	100 cc. Ca-free	—	0	0	0
	5	9·30	6·35	1·9	Ringer solution	20	5·90	5·90	1·8
VII	0	9·9	—	—	100 cc. Ca-free	—	0	0	0
	1½	9·9	—	—	Ringer solution	—	—	—	—
	3	9·9	6·7	—		45	6·4	6·4	—

Table X.

Exp.	Time in hours	Mg. Ca per 100 cc. serum		Amount of fluid introduced into peritoneal cavity	Mg. Ca per 100 cc. fluid in peritoneal cavity	
		Total	Diffusible		Total	Diffusible
VIII	0	11·40	6·25	100 cc. Ca-free	0	0
	1	—	—	Ringer solu-	3·15	—
	2	—	—	tion	5·50	—
	3	—	—		6·55	—
IX	0	9·15	5·45	120 cc. Ca-free	0	0
	1	—	—	Ringer solu-	2·60	—
	2	—	—	tion	3·40	—
	3	—	—		4·30	—
	4	—	—		5·25	—
	5	—	—		6·2*	—
	7	—	—		6·3*	5·40
X	0	10·1	7·6	100 cc. Ca-free	0	0
	1	—	—	Ringer solu-	3·27	—
	3	—	—	tion	5·30	—
	3½	—	—		7·10	—
	4	9·6	7·2		7·60	7·5

* Biuret test positive.

From the figures recorded in Tables IX and X, and those represented graphically in Fig. 1, it is evident that calcium diffuses from the circulating plasma through the peritoneal membrane into the fluid present in the peritoneal cavity. Exps. V–VII show that enough calcium has diffused into the

calcium-free fluid during a period of 5 hours to reach a concentration in the latter equal to the diffusible calcium of the serum. In Exps. I–IV, where Ringer solution having a calcium concentration of 8 mg. calcium per 100 cc. was used, there was a reduction in this concentration of such a degree that at the end of 5 hours the concentration was equal to that of the diffusible calcium in the serum. Conversely in Exps. III and IV, where a Ringer solution containing 5·65 mg. calcium per 100 cc. was used, this concentration was raised to a similar level. A study of Fig. 1 shows that the increase in the calcium concentration of originally calcium-free Ringer solution, allowed to remain in the peritoneal cavity, is rapid and steady for the first 3 hours, after which it diminishes and tends to remain constant when it approximately equals that of the diffusible fraction of the serum-calcium.

With the exception of one experiment the fluid recovered from the peritoneal cavity at the end of the experiment gave practically no biuret reaction, and any small amount of protein present could either be derived from the

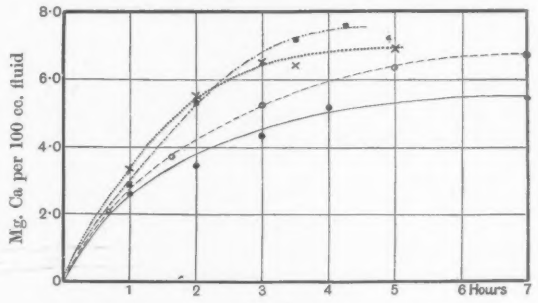

Fig. 1.

secretion of the membrane itself, or result from contamination with traces of blood. In Exp. IX, where the biuret test was definitely positive, the last samples were obviously contaminated with blood and in this case the diffusible calcium in the last sample was slightly lower than the total calcium, while in all the other experiments in which this estimation was carried out the whole of the calcium present was found to be diffusible.

The facts observed may be summarised as follows. In all cases a calcium-containing fluid, in which the calcium concentration equals that of the diffusible calcium of the serum, has been recovered from the peritoneal cavity, irrespective of whether the fluid originally contained either no calcium, or quantities of calcium in excess of, or slightly less than, the diffusible calcium of the serum. The curve of diffusion definitely tends to reach a constant level. The whole of the calcium which has entered the peritoneal cavity can be recovered in a biuret-free collodion ultra-filtrate and is therefore not in colloidal combination. Lastly, some of the calcium which has diffused through the peritoneal membrane is in the form of calcium ions.

It seems justifiable to conclude, therefore, that a fraction of the calcium present in the plasma exists as a non-colloidal diffusible complex which is partly in the ionised state, and that this diffusible complex and also the ionised calcium is equal in amount to that which is present in the corresponding serum.

Conversely these results strongly suggest that the distribution of calcium in the serum between crystalloid and colloid as determined by collodion ultrafiltration is quantitatively comparable with that in plasma.

From the evidence put forward in this paper it would appear that some calcium is withdrawn from the plasma during the process of clotting. Since the findings suggest that the amount of diffusible and ionised calcium in the plasma is equal to that of the serum, it seems probable that it is the non-diffusible complex which is involved.

Summary.

True plasma contains 10–20 % more calcium than does the corresponding serum. In citrated plasma the amount is less than in true plasma to an extent depending on the amount of citrate added.

The corpuscles contain no more than traces of calcium. In plasma the concentration of ionic and of diffusible calcium appears to be equal to that in serum. It is suggested that during the coagulation of blood some of the non-diffusible calcium is precipitated.

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LXXI. STUDIES IN CALCIUM METABOLISM.

III. CALCIUM AND THE COAGULATION OF BLOOD.

By CORBET PAGE STEWART and
GEORGE HECTOR PERCIVAL (*Grocers' Company's Research Scholar*).

From the Departments of Medical Chemistry, Pharmacology and Therapeutics, University of Edinburgh.

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It is commonly stated that calcium ions are necessary for the clotting of blood, a view which was apparently first put forward with definite evidence by Arthus and Pagès [1890] and later by Sabatani [1903]. It appears to depend firstly on the facts that blood coagulation is prevented by the addition of oxalates which precipitate calcium, and by citrates which lead to the formation of an almost undissociated calcium salt, and secondly on the fact that blood in which coagulation has been prevented by addition of oxalate can be made to clot by the further addition of soluble calcium salt.

Recently Wadsworth, Maltaner and Maltaner [1927] have put forward a theory of blood coagulation according to which the process is initiated by the reaction, at a surface of contact, between ionised calcium salts and an active lipoid, with formation of an ionised calcium lipoid compound and acid. The chemical reasoning on which this hypothesis is based does not seem very convincing, but the paper is interesting as insisting on the necessity for calcium ions.

So far no direct evidence has been produced in support of the statement that calcium in the ionised state is an essential factor in blood coagulation. We found that the addition of sodium citrate to blood produced an effect, additional to the formation of an undissociated calcium salt—it broke down the undissociated non-diffusible calcium complexes of the blood. Since the amount of citrate added to the blood was vastly in excess of the amount required to combine with the whole of the calcium present, it seemed probable that almost all the calcium was existing as calcium citrate. Thus the question of the form of combination in which calcium aids coagulation was thrown completely open, since the citrate had combined with the ionised calcium, and probably with the non-ionic but diffusible calcium, as well as with that derived from the breakdown of the non-diffusible complexes.

In these circumstances it was thought desirable to investigate quantitatively the effect of addition of citrate to the blood on the various forms of combination of calcium and on the coagulability.

Experiments with citrated blood.

The amounts of sodium citrate recommended in text-books for the purpose of preventing blood clotting are undoubtedly greater than are really necessary, and we therefore first investigated the effect of varying amounts of sodium citrate on the coagulability of blood. Through the kindness of Mr Goffin we were afforded facilities for carrying out these and subsequent experiments at the municipal abattoir, using ox blood. As soon as the animal's throat was cut, a sample was collected, and 2 cc. were rapidly measured into each of a series of test-tubes containing varying amounts of solid sodium citrate. (Measured volumes of a standard sodium citrate solution were added to the tubes and evaporated to dryness.) The tubes were shaken at once to dissolve the citrate and obtain thorough mixing, and the coagulation time was measured from the time the sample was collected to the time when the tube could safely be inverted. A further sample from the same animal was set aside, and allowed to clot, the serum being taken for analysis. A series of serum samples was set up with sodium citrate added in concentrations equivalent to those used in the coagulation series, calculated on the assumption that the serum represented 60 % of the whole blood. In these samples total calcium, diffusible and ionic calcium were determined. The total calcium and diffusible calcium were determined as described in the preceding papers of this series. For the determination of ionic calcium the method of Brinkman and Van Dam [1920] was employed. In this method, 1 cc. of the sample is added to each of a number of small tubes containing varying amounts of solid sodium oxalate. After standing for 1 hour, the tubes are examined, and note is made of that tube which is just clear, and that which is just turbid owing to precipitation of calcium oxalate. The solubility product of $Ca^{\cdot\cdot}$ and C_2O_4'' being known, it is then possible, since the concentration of C_2O_4'' is known, to calculate the concentration of $Ca^{\cdot\cdot}$ with a degree of accuracy depending on the variations in the C_2O_4'' concentration of successive tubes. In practice the error is about 15 %. The differences observed, however, were so great as to be beyond even this experimental error. In dealing with serum it was often impossible to obtain any satisfactory result owing to the opalescence of the serum itself, but it was found that, as Brinkman and Van Dam state, those sera from which results could be obtained invariably gave values identical with those obtained from their clear ultra-filtrates. Throughout this work, therefore, the serum filtrate was employed in the estimation of ionic calcium. Table I shows the effect of sodium citrate on the coagulation time of ox blood, while Table II shows the effect of the salt, in corresponding concentrations, on the total, diffusible, and ionic calcium content of the sera from these same bloods. One case is shown graphically in Fig. 1. From these results several facts emerge. In the first place, it is evident that no precipitation of calcium takes place when sodium citrate is added in amounts just necessary to prevent coagulation, though considerably higher concentrations do produce such a precipitation.

Sodium citrate g. per 100 cc. serum
0·00
0·01
0·02
0·03
0·04
0·05
0·06
0·07
0·08
0·09
0·10
0·20
0·30
0·40
0·60
0·80
1·00
1·50
2·00
5·00

Table I. *Effect of sodium citrate on the coagulation time of ox blood.*

Sodium citrate g. per 100 cc.		Coagulation time at 18°						
Blood	Serum	1	2	3	4	5	6	7
0·00	0·00	5′ 13″	8′ 45″	5′ 46″	5′ 5″	9′ 0″	8′ 45″	3′ 50″
0·00	0·00	5 6	8 50	5 36	5 0	8 50	8 15	3 55
0·01	0·017	4 20	7 30	4 30	5 10	—	8 15	4 15
0·02	0·033	4 17	—	5 40	—	—	—	—
0·025	0·042	—	—	—	5 30	7 20	8 35	3 55
0·03	0·050	3 57	8 30	—	—	—	—	—
0·05	0·083	4 5	8 20	6 55	6 45	9 0	6 35	4 25
0·10	0·17	—	—	—	13 20	13 0	12 20	8 10
0·125	0·21	—	—	—	21 30	27 0	13 15	12 0
0·15	0·25	15 22	11 0	16 11	45 0	35 0	22 0	17 0
0·20	0·33	No coag.	No coag.	No coag.	No coag.	No coag.	No coag.	No coag.
0·25	0·42	,,	,,	,,	,,	,,	,,	,,
0·40	0·66	,,	,,	,,	,,	,,	,,	,,
0·50	0·83	,,	,,	,,	,,	,,	,,	,,

Table II. *Effect of sodium citrate on the total, diffusible, and ionic calcium of ox serum.*

Sodium citrate g. per 100 cc. serum	Total calcium mg. per 100 cc. serum					Diffusible calcium mg. per 100 cc. serum					Ionic calcium mg. per 100 cc. serum				
	3	4	5	6	7	3	4	5	6	7	3	4	5	6	7
0·00	9·50	12·00	10·05	11·30	11·30	4·55	5·80	5·30	5·60	6·45	2·5	2·5	2·4	2·3	2·16
0·01	9·50	—	10·00	—	—	4·55	6·00	5·20	—	—	1·5	1·4	1·25	1·4	—
0·02	—	—	—	—	—	4·60	5·80	5·25	5·80	—	—	—	—	—	—
0·03	—	12·00	—	—	—	—	5·80	5·25	—	—	—	—	—	—	—
0·04	—	—	—	—	—	4·75	6·05	5·30	—	—	—	—	—	—	—
0·05	9·50	—	—	—	—	5·20	6·40	5·25	5·70	6·25	0·7	0·43	0·72	0·45	1·08
0·06	—	12·00	—	—	—	5·50	6·50	5·25	—	—	—	—	—	—	—
0·07	—	—	10·00	—	—	5·90	6·70	5·50	—	—	—	—	—	—	—
0·08	—	—	—	—	—	6·35	7·00	5·70	—	—	—	—	—	—	—
0·09	—	12·00	—	—	—	—	7·20	6·30	—	—	—	—	—	—	—
0·10	—	—	—	—	—	6·80	7·30	7·25	8·10	7·55	—	0·36	0·27	0·27	0·36
0·20	—	—	—	—	—	—	—	—	10·10	8·65	—	—	—	—	—
0·30	—	12·10	—	—	—	8·45	9·60	9·05	11·00	9·70	—	—	0·24	0·24	0·27
0·40	—	—	—	—	—	8·80	—	9·30	10·90	10·40	—	—	—	—	—
0·60	—	—	—	—	—	9·20	10·90	9·45	11·10	11·30	—	—	>0·21	>0·21	0·21
0·80	—	—	—	—	—	9·20	11·50	9·50	—	—	—	—	—	—	—
1·00	9·60	12·00	10·00	—	—	9·10	11·50	9·45	—	—	>0·21	>0·21	>0·21	>0·21	>0·21
1·50	9·40	11·80	10·00	—	—	8·90	11·10	9·45	—	—	—	—	—	—	—
2·00	8·70	10·80	10·00	—	—	8·20	9·60	9·05	—	—	—	—	—	—	—
5·00	5·50	8·00	6·05	—	—	5·10	7·80	5·50	—	—	—	—	—	—	—

It is thus evident that, as is usually stated, sodium citrate does not owe its anti-coagulant power to any removal of calcium from the blood. Secondly, it may be noted that sodium citrate, even in very low concentrations, undoubtedly causes a serious diminution in the amount of ionic calcium in the blood—presumably by the formation of calcium citrate which is commonly supposed to be almost undissociated, a fact which the authors have confirmed by measurements of the conductivity of calcium citrate solutions. It is obvious, however, that the diminution of calcium ions cannot be the cause of the loss of coagulability, for at a point when their concentration has been reduced to one-tenth of the original amount, the blood coagulates at the normal rate, and, in a number of cases, even more rapidly than when no citrate is present. Moreover, increased amounts of citrate have very little effect on the remaining

ionic calcium. The third fact, which is shown in Tables I and II, and in Fig. 1, is that with those concentrations of citrate which are without effect on the rate of coagulation (or slightly accelerate it) the diffusible calcium remains at the normal level, but coincident with the lengthening of the coagulation time there is an increase in the diffusible calcium, which becomes nearly equal to the total, precisely at that concentration of citrate which completely prevents coagulation from taking place. Regarded from another angle, it is seen that whereas the almost total loss of the ionised fraction of the calcium is without effect on the coagulation time, the loss of the non-diffusible calcium is accompanied by a lengthening of the coagulation time, and that loss of coagulability

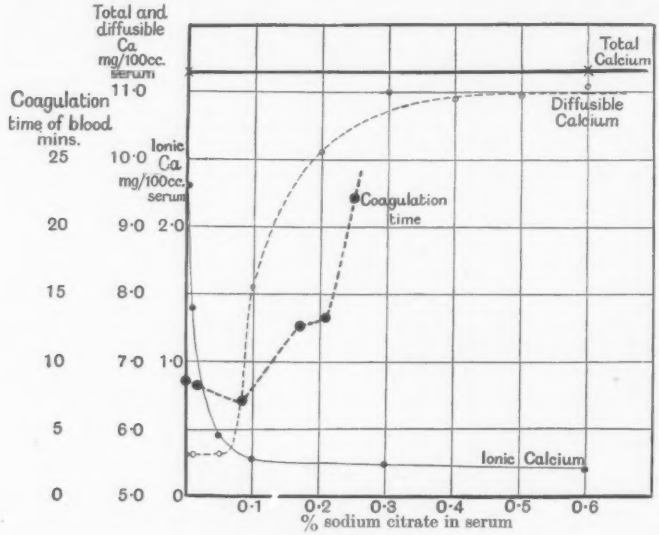

Fig. 1.

coincides with the reduction of this non-diffusible calcium to a very low level. The conclusion is inevitable—provided the validity of the data be granted—that neither the ionic nor the simple undissociated compounds of calcium are necessary for coagulation, but that, as Vines [1921] has deduced from other considerations, some complex, undissociated calcium compound is.

In view of the proviso with which this conclusion is accompanied, it is necessary to point out in what way the experimental data may be held insufficient to justify the deductions drawn from them, and to give the reasons for which, in the authors' opinion, they are to be considered capable of supporting these deductions. The analytical figures themselves are of sufficient accuracy, for experience of the Kramer-Tisdall method extending over several years has convinced us of its reliability, and its capability, with careful work, of yielding results with an error of not more than 2 %. In the method used

for determining the ionic calcium the error is much greater, but the changes are so gross, and occur with such unfailing regularity that no doubt remains of their essential reality. The same is true of the measurement of coagulation time; little reliance can be placed on small changes, but the method readily detects real changes of considerable magnitude. Criticism may, however, be directed to the fact that we have compared the effect of sodium citrate on the coagulation time of blood with the effect on the calcium content of the serum instead of the plasma. Of course, it was impossible to use plasma, which would have clotted long before collodion filtration was complete. The use of anti-coagulants, obviously, was equally impossible and serum presented the only possibility. The objection to its use lies in the suggestions which have been made (*e.g.* by Vines) that in the distribution of calcium serum differs from plasma. It is true that, as the authors have themselves shown, serum contains less calcium than does true plasma, but in the same paper they have advanced arguments, derived from their own and others' work, in favour of the idea that calcium is similarly distributed in plasma and in serum. If this idea be accepted, the objection to the use of serum in the experiments here described falls to the ground. Even if it be rejected, it may still be argued that though the distribution may be quantitatively different in the two fluids, it must be qualitatively similar, in which case the data from serum analysis remain sufficient for their present purpose. For example, were the concentration of ionic calcium greater in plasma than in serum, it would be fair to suggest that the calcium ions disappear from serum with a concentration of citrate so low in comparison with that required to prevent coagulation, that there is ample "margin of safety." Similarly, if it be supposed that the concentration of non-diffusible calcium be higher in plasma than in serum, there is no doubt that citrate will equally in plasma, as in serum, produce a considerable diminution in this fraction. If it be agreed that the use of serum is justified, then the conclusions drawn from the experiments set out in Tables I and II are valid; if not, they are untenable, as also are those drawn from the further experiments yet to be detailed.

Reverting for a moment to the implications of the experiments with sodium citrate, a further point of some interest may be mentioned, although it will be dealt with more fully at a later stage. In several cases it was noticed that blood containing a small amount of citrate clotted more rapidly than normal blood. At these concentrations the amount of diffusible (and therefore of non-diffusible) calcium was normal; only the amount of ionic calcium had been altered. One may enquire whether calcium ions are not only unnecessary for blood coagulation, but actually have some inhibiting effect. This suggestion is made with great reserve for, as has already been mentioned, the method employed for the determination of the coagulation time is not one which can be relied upon in dealing with small changes. Moreover, the drop in the coagulation time was not observed in all cases, though there was never an increase with very low concentrations of citrate. Still, it is striking that in

those cases where acceleration of clotting did occur, the return to normal coincided with the point at which the concentration of ionic calcium reached a low level whence it decreased only slowly with increased concentration of citrate. This coincidence is well shown in Fig. 1.

Experiments on the recalcification of citrated blood.

The addition of calcium chloride to citrated blood restores the power of coagulating, and if the conclusions reached in the first series of experiments are correct, recalcification should bring about an increase in the non-diffusible calcium of the blood. The case will be still further strengthened if coagulation takes place at a point where non-diffusible calcium is present in quantity while ionic calcium is still almost absent. In order to determine whether this was the case, ox blood was prevented from clotting by the addition of sodium citrate in amount corresponding to 0·42 g. per 100 cc. plasma. Two hours after citration it reached the laboratory, and then 2 cc. were added to each of a series of test-tubes containing known amounts of dry calcium chloride. After thorough mixing, the tubes were allowed to stand and the coagulation times observed. A second sample of blood from the same animal was allowed to clot, the serum was mixed with sodium citrate (0·42 g. per 100 cc.), and allowed to stand for 2 hours. To a series of samples calcium chloride was added in the concentrations used for the citrated blood, and determinations of the total and diffusible calcium were made. As in all experiments described in this paper, the serum was centrifuged before being sampled for analysis.

Table III. *Effect of recalcification on the coagulability of citrated blood and on the calcium content of citrated serum.*

Coagulation time of blood with equivalent treatment		Total calcium mg. per 100 cc. serum			Diffusible calcium mg. per 100 cc. serum			Non-diffusible calcium mg. per 100 cc. serum		
		II	III	IV	II	III	IV	II	III	IV
11′	Normal serum	9·0	9·75	10·8	3·9	5·70	4·6	5·1	4·05	6·2
No coagulation	Serum + 0·42 g. sod. cit. per 100 cc.	8·6	9·10	10·1	8·1	9·10	10·0	0·5	0·00	0·1
No coagulation	Citrated serum + 8·3 mg. Ca per 100 cc.	17·0	18·25	18·4	16·2	17·0	17·9	0·8	1·25	0·5
>6<18 hours	Citrated serum + 16·6 mg. Ca per 100 cc.	24·9	26·30	26·6	21·9	23·85	24·3	3·0	2·45	2·3
20′	Citrated serum + 33·2 mg. Ca per 100 cc.	36·6	38·20	40·0	33·3	33·60	34·8	3·3	4·60	5·2
10′	Citrated serum + 49·8 mg. Ca per 100 cc.	47·0	49·30	50·0	41·4	43·2	44·1	5·6	6·10	5·9

The results of experiments of this kind are shown in Table III. Addition of considerable quantities of calcium was necessary in order to restore coagulability. This, of course, was to be expected in view of the relatively huge amount of citrate added, which, assuming the salt used to be tri-sodium citrate, was chemically equivalent to 96 mg. of calcium. The table shows quite clearly, however, that with the return of coagulability there is a coincident increase in the amount of non-diffusible calcium, and that this amount approaches the normal level just as the coagulation time approaches the normal. Not only is this the case, however, but the concentration of calcium ions is also increased by recalcification, and, moreover, this increase, like that in the

non-diffusible calcium, coincides with the return of coagulability. Considered alone, therefore, the recalcification experiments do not permit of any deduction as to the necessity of ionic calcium or of non-diffusible calcium. In the experiments on the direct citration of blood, however, there was so striking a reduction—amounting almost to a disappearance—of the calcium ions at concentrations of citrate which were totally without effect on the coagulation rate, that further evidence of the non-essential nature of calcium ions in blood clotting is hardly required. If then it be granted that the ions are not a factor in coagulation, the recalcification experiments do afford supporting evidence for the hypothesis that non-diffusible calcium is required.

Experiments with sodium fluoride as anti-coagulant.

The methods employed in this and the following section need not be detailed since they were essentially the same as those used in the case of citrated blood. The results (Tables IV and V and Figs. 2 and 3) lead to the same conclusions as were reached when sodium citrate was the anti-coagulant. There is a rapid fall in the ionic calcium, which reaches a very low level at a fluoride concentration without any retarding action on coagulation. There is again a reduction in the non-diffusible calcium, noticeable only when the coagulation time begins to increase, and at a concentration of fluoride sufficient to prevent coagulation the non-diffusible calcium has been reduced to a small fraction of its original amount. The final result, though identical with that produced by citrate, is, however, reached by a different route. In the case of citrate,

Table IV. *Effect of sodium fluoride on the coagulation time of ox blood.*

Sodium fluoride g. per 100 cc.		Coagulation time at 18°		
Blood	Serum	XVII	XXIII	XL
0	0	6′ 40″	6′ 15″	10′ 20″
0	0	6 45	6 10	10 30
0·09	0·15	6 40	6 20	10 30
0·12	0·20	2 45	5 55	9 50
0·15	0·25	4 50	5 0	7 15
0·18	0·30	5 40	6 35	11 20
0·22	0·37	—	8 50	18 0
0·26	0·43	20 0	—	22 40
0·30	0·50	30 0	14 30	30 0
0·36	0·60	No coag.	No coag.	No coag.

Table V. *Effect of sodium fluoride on the total, diffusible, and ionic calcium of ox blood.*

Sodium fluoride g. per 100 cc.		Total calcium mg. per 100 cc.				Diffusible calcium mg. per 100 cc.				Ionic calcium mg. per 100 cc.			
Blood	Serum	XVII	XXII	XXIII	XL	XVII	XXII	XXIII	XL	XVII	XXII	XXIII	XL
0·00	0·00	9·60	10·60	10·50	10·4	4·80	5·60	6·30	4·4	2·88	2·4	2·50	2·50
0·012	0·02	9·45	—	—	—	4·25	5·17	5·60	—	1·66	—	1·40	1·50
0·03	0·05	9·25	—	—	—	3·65	4·15	5·05	—	0·54	—	0·54	0·54
0·06	0·10	7·7	—	—	8·4	2·90	3·00	3·13	3·2	0·31	—	0·36	0·31
0·12	0·20	6·9	—	—	6·7	1·30	—	—	2·4	0·21	—	—	—
0·18	0·30	5·50	—	—	5·4	0·70	0·66	0·60	1·8	>0·21	>0·24	>0·21	>0·21
0·24	0·40	—	—	—	4·8	—	—	—	1·3				
0·36	0·60	1·00	1·80	0·70	4·0	0·15	0·30	0·20	1·1	>0·21	>0·21	>0·21	>0·21

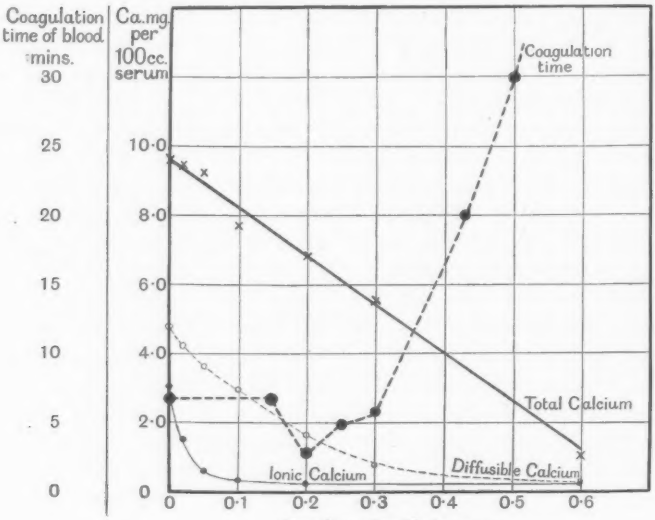

Fig. 2.

In all figures:

●---● Coagulation time. ×——× Total calcium.
o- - - -o Diffusible calcium. •——• Ionic calcium.

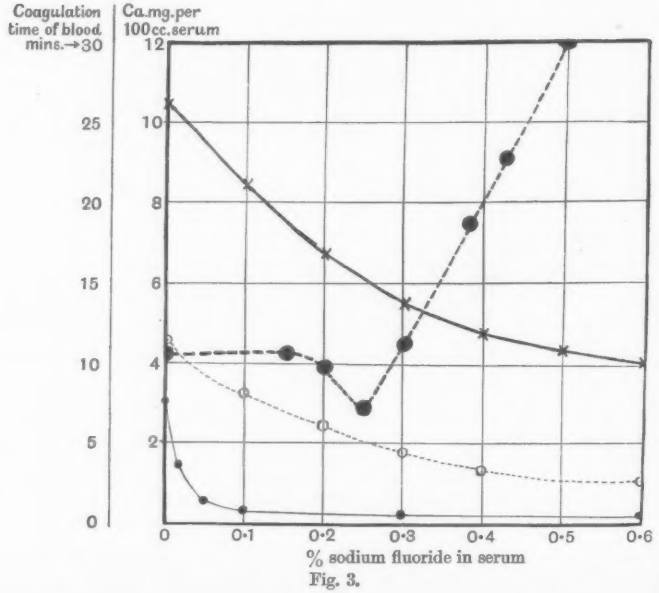

Fig. 3.

almost the whole of the calcium formed some diffusible but almost un-ionised compound, presumably calcium citrate; in the case of fluoride, calcium is precipitated, and with low concentrations of fluoride, diffusible calcium is preferentially precipitated.

Addition of fluoride, like addition of citrate, to blood produces that curious phenomenon, an acceleration of clotting in presence of low concentrations of the anti-coagulant. Again the decrease in coagulation time coincides with the removal of the greater part of the calcium ions, and its return to normal with the appearance of the second effect of the salt—the decrease in the non-diffusible calcium. When, however, the analytical results were considered in further detail, it was noticed, in those first treated, that throughout the range of fluoride concentrations producing this improved coagulability there was actually a slight increase in the non-diffusible calcium. This is shown in Fig. 2 (Serum XXII), where the diffusible calcium values lie very well on a curve convex with respect to the straight line representing the corresponding values for the total calcium. This result, however, appears to be entirely fortuitous, for though it has been found in many cases, in others, almost equally numerous, the two curves, as in Fig. 3, are almost parallel, though slightly converging, from the start. Since, even in these cases, the blood showed the usual decrease in the coagulation time, there can be no causative relation between the coagulability and the increase in the non-diffusible calcium. It is difficult to explain the latter satisfactorily, though one may perhaps suggest an adsorption of calcium fluoride. In attempting to explain the more rapid clotting in presence of small amounts of fluoride, one is thus driven, as in the case of citrate, to suggest an actual inhibitory effect due to the calcium ions. The only alternative seems to be a slight alteration in p_H due to the presence of the anti-coagulant. This possibility has not been considered experimentally, but it seems hardly likely, at any rate, that the two substances sodium citrate and sodium fluoride would alter the p_H in the same direction.

Experiments with sodium chloride as anti-coagulant.

Sodium chloride prevented blood coagulation at a concentration of 1·8 g. per 100 cc. With the serum concentration corresponding to this, the non-diffusible calcium was again markedly reduced (though not to the extent observed in the case of citrate) by an increase in the amount of diffusible calcium (Table VI and Fig. 4). Once more there was a decrease in the coagulation time with low concentrations of anti-coagulant, and an early decrease in the concentration of calcium ions. The disappearance of ionic calcium was, however, slight when compared with that in the previous series of experiments, and indeed over 50 % of the original concentration remained in sera corresponding to uncoagulable blood. Generally, the picture is very similar to that seen in the case of citrate, but is less strongly marked. From it alone no definite deductions could be drawn, but taken in conjunction with the other series, it affords confirmatory evidence of the necessity, in blood coagulation, of non-diffusible calcium.

Table VI. *Effect of sodium chloride on the coagulation time of ox blood and on the total, diffusible and ionic calcium of the serum.*

Sodium chloride g. per 100 cc.		Coagulation time		Total calcium mg. per 100 cc.		Diffusible calcium mg. per 100 cc.		Ionic calcium mg. per 100 cc.	
Blood	Serum	XX	XXI	XX	XXI	XX	XXI	XX	XXI
0·00	0·00	12′ 10″	9′ 10″	9·75	11·0	5·60	6·10	1·80	2·16
0·60	1·0	8 45	6 50	—	—	6·00	7·65	1·66	1·20
0·90	1·5	9 0	6 55	—	—	6·35	—	1·66	—
1·20	2·0	7 15	5 50	—	—	6·70	—	1·44	—
1·50	2·5	15 0	—	—	—	—	—	—	—
1·80	3·0	No coag.	No coag.	—	—	7·20	8·65	1·44	1·08
2·40	4·0	,,	,,	9·80	11·1	8·35	9·05	1·08	0·72
3·60	6·0	—	—	—	—	—	9·35	—	0·54

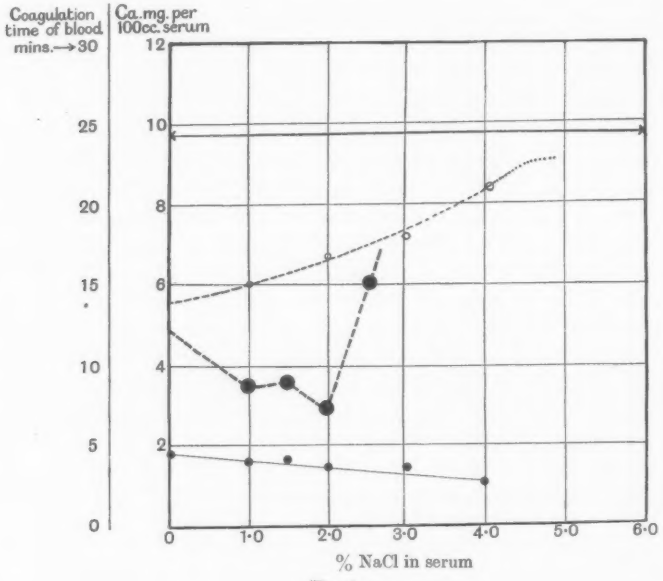

Fig. 4.

EXPERIMENTS WITH SODIUM OXALATE AS ANTI-COAGULANT.

In the experiments so far recorded, one factor was ignored; the time required for an equilibrium to be reached after the addition of the salt to blood or serum. Whether or not this omission is serious depends on the rate at which the equilibrium is adjusted in response to the addition of anti-coagulant. The essential action of the anti-coagulant takes place within some 5 or 10 minutes, the normal time of coagulation; the time required for filtration of serum is 2 hours. It has been tacitly assumed that the analyses made after the lapse of this preliminary filtration period give figures which represent the position in the serum 5 minutes after the addition of the anti-coagulant. It must be admitted that the neglect to take this factor into account was an oversight,

but there is some evidence that in the cases so far considered it is not of great importance. Thus, in the experiments on aerated plasma described in the previous paper it was found that precipitation of calcium occurred within 6 minutes of the addition of sodium citrate, and was not appreciably greater after a lapse of 2 hours. The precipitation of calcium by fluoride also appeared to be rapid since samples of serum centrifuged $\frac{1}{2}$ hour and 2 hours after the addition of sodium fluoride were found to have identical calcium contents. In any case, so long as the changes proceed at a moderate rate, the effect of making the determinations after 2 hours (as the available methods render necessary) can only be to exaggerate somewhat the quantitative action of the anti-coagulant. Hence the omission of the time factor does not invalidate the conclusions drawn from these experiments.

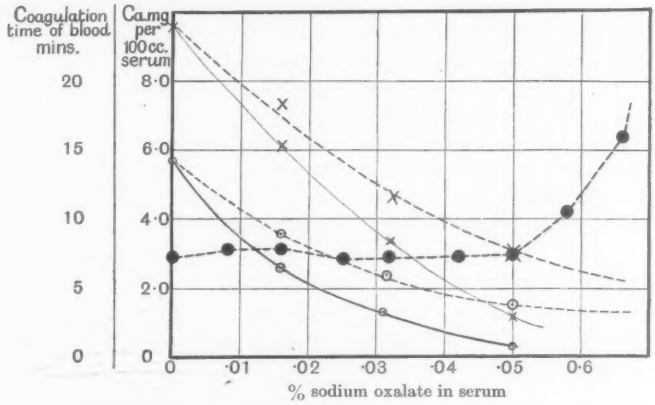

Fig. 5.

In dealing with sodium oxalate, however, an attempt has been made to take the time factor into account. To this end the filtration of the serum was, as usual, commenced immediately after addition of sodium oxalate. Enough filtrate for analysis was obtained after 2 hours. The filtrate was centrifuged before withdrawal of the sample. Now the precipitation of calcium by oxalate is not complete in 2 hours, but experiments reported in the preceding paper of this series showed that with small quantities of oxalate the amount of calcium precipitated in 120 minutes was approximately 1·33 times the amount precipitated in 10 minutes. Hence it was possible to calculate, approximately, the amount of diffusible calcium present in the serum 10 minutes after the addition of the anti-coagulant. For the determination of total calcium, the serum was centrifuged for 2 minutes, commencing 8 minutes after the addition of the oxalate so that samples for analysis were obtained after the re-adjustment of the equilibrium had been in progress for 10 minutes. To test the correctness of this method of calculation, the serum remaining after withdrawal of samples for analysis was again centrifuged after the lapse of 1 hour,

and a second series of samples was withdrawn for analysis. The amount of calcium precipitated in 1 hour is approximately 1·25 times the amount precipitated in 10 minutes, and calculation on this basis from the results obtained in the second series gave values in good agreement with those obtained by direct determination. For routine purposes, therefore, the whole serum was centrifuged 1 hour after the addition of the sodium oxalate and the results for a 10 minutes' period were then calculated in the same way as those for the diffusible calcium. Determination of ionic calcium was not attempted. The results are shown graphically in Fig. 5, where the continuous lines represent the actual figures obtained by analysis, and the dotted lines the figures obtained by calculation representing approximately the position 10 minutes after the addition of the sodium oxalate. As in the series of experiments previously described, there is a progressive decrease in the concentration of non-diffusible calcium, and it is only when this concentration becomes very small that coagulation is delayed and finally prevented. The experiments thus lead to exactly the same conclusions as do those with other salts with an anticoagulant action.

Summary.

From experiments on the effect of sodium citrate, sodium fluoride, sodium chloride, and sodium oxalate on the coagulation time of ox blood and on the concentration of calcium, diffusible calcium, and ionic calcium of the corresponding serum, it is concluded that some complex calcium compound, incapable of filtration through a collodion membrane, is essential for the coagulation of blood. Calcium ions are not necessary, and it is tentatively suggested that they may actually have an inhibitory effect.

One of us (C.P.S.) is in receipt of a personal grant from the Medical Research Council. The expenses of the work were partly defrayed by a grant from the Moray Research Fund of this University.

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LXXII. THE DETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS.

By RONALD KINGTON CHRISTY and WILLIAM ROBSON.

From the Department of Physiology, King's College, London.

(*Received February 4th, 1928.*)

Biochemical methods for estimating chlorides in present day use are, with the exception of those of Bang [1913] and McLean and Van Slyke [1915], all modifications of the Volhard technique.

McLean and Van Slyke state that the least possible quantity of halide that can be estimated accurately by Volhard's method is 0·5 millimol., and, in order that they might estimate smaller quantities than this, they evolved an iodometric method. According to this technique potassium iodide is used to titrate the excess of silver nitrate, the end-point being determined by a sodium nitrite-starch indicator functioning in an acid solution buffered by the addition of trisodium citrate.

From the point of view of the sharpness of the end-point thus obtained, the method was a decided advance. Nevertheless it has two inherent disadvantages. The nitrous acid-starch-iodine indicator functions correctly only within a very limited range of p_H and if, by any chance, the amount of citrate added does not reduce the acidity of the solution within that range, the true colour associated with the iodine-starch combination is not produced. Moreover an appreciable amount of potassium iodide in excess of the theoretical requirement is necessary for the development of colour at the end-point.

One of us (W.R.), recently collaborating with Dr Van Slyke, substituted other buffers for trisodium citrate in an attempt to widen the range of p_H within which the blue colour of the indicator is produced. This, however, did not give the desired increase in accuracy.

The essential chemical reaction involved at the end-point in the method of McLean and Van Slyke is the liberation of iodine by nitrous acid from the excess of hydrogen iodide present in the solution. A similar liberation of iodine takes place when potassium iodate and potassium iodide react in acid solution, and this was utilised by Bang [1913] in his method of estimating halides. On applying this reaction in the presence of starch to the titration of the excess of silver nitrate (in acid solution) with potassium iodide it was found that an appreciable excess of the last named reagent was again necessary for the development of the blue colour. Replacement, however, of the potassium iodate by potassium bi-iodate, $KH(IO_3)_2$, leads to the

instantaneous production of colour once the slightest excess of iodide is present in the solution. The equation for the reaction involved, as given by Friend [1924], is $KIO_3.HIO_3 + 10KI + 11HNO_3 = 11KNO_3 + 12I + 6\,H_2O$.

Experimental results show that this reaction can take place, apparently with equal speed, over a wide range of hydrogen ion concentration (*vide* Table I). The buffering of solutions containing that amount of nitric acid usually added for the precipitation of halide is therefore unnecessary. Thus both the disadvantages of the original method of McLean and Van Slyke are met.

By the proposed method so small a quantity as 2·5 mg. of sodium chloride has been accurately estimated, and the combined sensitivity and sharpness of the end-point make it likely that even smaller quantities than this may be estimated if use were made of the micro-titration technique of Rehberg [1925].

Method.

Materials required.

The reagents are $N/29{\cdot}25$ solutions of silver nitrate and potassium iodide, 1 cc. of either of which is equivalent to 2 mg. of sodium chloride; concentrated nitric acid free from chloride; a $N/100$ solution of potassium bi-iodate in distilled water; and a 1 % solution of soluble starch. The last solution should not be kept longer than a fortnight.

Experimental procedure.

In the analysis of fluids containing protein the proposed method consists of three steps: (1) removal of protein, (2) precipitation of chloride, and (3) final titration. For fluids containing no protein only the last two steps are necessary.

(1) *Removal of protein.* One of the several advantages of the proposed method is its applicability to any of the ordinary protein-free blood filtrates in present-day general use. In the present investigation both the Folin and Wu [1919] filtrate (acid precipitant), and the Hagedorn and Jensen [1923] filtrate (alkaline precipitant), have been employed with success.

(2) *Precipitation of chloride.* To every 20 cc. of solution (diluted urine or blood-filtrate), 0·5 to 1·0 cc. of nitric acid is added, and the chloride is precipitated by excess of the standard silver nitrate solution. Complete flocculation of the silver chloride requires a few minutes, after which the mixture is made up to a definite volume and filtered. Filtration is essential in order to prevent the occurrence of the reaction $AgCl + KI = AgI + KCl$ during the next step.

(3) *Final titration.* To an aliquot portion of the filtrate from the previous stage is added 1 cc. of each of the solutions of potassium bi-iodate and soluble starch, and the standard potassium iodide is finally run in from a burette. The blue end-point is very sharp.

Experimental results.

1. *The effect of p_H on the development of the blue colour at the end-point.*

Table I.

Amount of NaCl present = 0·103 g. %

Amount of 50 % KAc added to the HNO_3 solution (cc.)	Approximate p_H attained	NaCl found (g. %)
0	2·0	0·102
		0·103
		0·103
0·5	3·0	0·103
		0·103
		0·103
1·0	4·0	0·103
		0·103
		0·103
2·0	5·0	No blue colour developed

Table II shows the results obtained by three methods, the authors' method, that of Whitehorn [1920] and that of Van Slyke [1923].

Table II.

Sodium chloride present = 0·103 g. %

Method	NaCl found (g. %)			
Authors	0·103	0·103	0·102	0·103
Whitehorn	0·106	0·107	0·106	0·106
Van Slyke	0·100	0·099	0·100	0·098

2. *Analysis of urine and recovery of* NaCl *added to urine.*

Table III shows the results of a series of analyses on a sample of urine to which sodium chloride in known amounts was added.

Table III.

NaCl added to 100 cc. urine (g.)	NaCl found (g. %)	Recovery (%)
0·0	1·10	—
0·1	1·200 (3)	100·3
0·15	1·251 (2)	100·6
0·20	1·301 (0)	100·5

3. *Analysis of blood.*

Table IV shows the results obtained by different methods on whole blood. The authors' method was carried out on two different blood-filtrates. The results (i) were obtained on the Hagedorn and Jensen filtrate, and results (ii) on the Folin and Wu filtrate. In each case the amount of filtrate used was equivalent to 0·5 cc. of blood, while in the other methods 1 cc. of blood was used.

Table IV.

Method	NaCl found (g. %)		
Authors (i)	0·455	0·455	0·456
Authors (ii)	0·456	0·456	0·456
Van Slyke	0·483	0·482	0·480
Whitehorn	0·421	0·421	0·421

Table V shows the recovery of NaCl, added to whole blood, by the methods applied above.

Table V.

Sodium chloride added g.	Sodium chloride recovered by the method of			
	Whitehorn	Van Slyke	Authors (i)	Authors (ii)
0·026	0·027	0·024	0·026	0·026
0·053	0·056	0·050	0·054	0·053
0·105	0·111	0·099	0·106	0·104
0·210	0·222	0·198	0·212	0·211
0·315	0·322	0·297	0·313	0·316
0·420	0·443	0·396	0·422	0·422

From the results tabulated above it will be seen that the error involved in the recovery of sodium chloride added to urine or to blood was at no time greater than 1 % and in most cases was less than this.

SUMMARY.

A new iodometric method of estimating chlorides in biological fluids is described. It is applicable to all cases in which the Volhard technique is used, and especially to blood-filtrates, *e.g.* those of Folin and Wu or Hagedorn and Jensen. The error involved in the method is less than 1 %.

One of us (W.R.) desires to acknowledge his indebtedness to the Medical Research Council for a part-time grant.

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LXXIII. THE PHOTO-OXIDATION OF CERTAIN ORGANIC SUBSTANCES IN THE PRESENCE OF FLUORESCENT DYES.

By CYRIL WILLIAM CARTER.

From the Department of Biochemistry, University of Oxford.

(*Received February 13th, 1928.*)

Introduction.

The present work was undertaken to extend the recent investigations of Harris, Gaffron, and others on the photo-dynamic oxidation of certain organic substances and to establish new data on the dynamics of this type of reaction. It was hoped that the information thus obtained might provide a basis for the explanation of the skin lesions and other manifestations exhibited by individuals sensitised to light by the occurrence in their tissues of certain fluorescent porphyrins. Fischer [1924], Garrod [1922] and Gray [1924] have reported in detail cases of haematoporphyrinuria congenita in which uro- and copro-porphyrin can be identified in the organs and excreta. Hausmann [1926] has described the symptoms produced in man and in small animals by injections of small doses of haematoporphyrin on exposure to light. Raab [1903], Tappeiner and Jodlbauer [1905], Dreyer [1904], Ledous Lebard [1902] and others have produced analogous effects on protozoa, bacteria, enzymes, toxins and immune bodies by the action of various fluorescent synthetic dyes in the presence of light. Harris [1926] has shown that proteins such as egg-albumin, haemoglobin and serum-albumin, and amino-acids like tyrosine and tryptophan undergo photo-oxidation when exposed to ultra-violet light and that this oxidation is greatly accelerated by the presence of minute traces of haematoporphyrin and other fluorescent dyes. Gaffron [1926] has investigated the photo-oxidation of tyrosine, phenol and uric acid using haematoporphyrin as sensitiser in visible light.

Two main facts emerge from the work of these and other authors. Firstly, fluorescence appears to be an essential physical property of those dyes which exhibit photo-dynamic activity. Thus Tappeiner [1909], in studying a long series of dyes, found that only those possessing the property of fluorescence were toxic to *Paramoecium* in light. Similarly Gaffron showed that while haemin, and the copper and silver salts of haematoporphyrin, which are non-fluorescent, are inactive as sensitisers for egg-albumin, the fluorescent zinc salt is active. The writer has shown that turacin, the non-fluorescent copper salt of uroporphyrin,

is inactive as a sensitiser for *Glaucoma* and tyrosine, whereas uroporphyrin, itself powerfully fluorescent, is an efficient sensitiser. At the same time there is reason for believing that the degree of fluorescence bears no direct relation to that of photo-dynamic activity. Raab [1903] showed that the fluorescent dye aesculin in concentration 1 : 1000 was without action on *Paramoecium*. The blue fluorescent substances described by Peters, Kinnersley and Squires [1925] in animal tissues are unable to bring about the photo-oxidation of tyrosine, and do not protect *Glaucoma* against the lethal action of haematoporphyrin in light. Quinine sulphate in aqueous solution gives a brilliant blue fluorescence in ultra-violet light but shows no absorption bands or fluorescence in visible light and is unable to bring about the photo-oxidation of tyrosine.

There is, secondly, the relationship of oxygen to the photo-dynamic process to be considered. Tappeiner came to the conclusion that in this respect ultra-violet and visible light differed in their photo-chemical action. Ultra-violet light possessing a high energy value is able to bring about a molecular rearrangement in living cells, enzymes and simple chemical compounds without the aid of oxygen. In the presence of oxygen these decomposition products are then oxidised, enabling the reaction to proceed to completion. In all probability this is effected by the agency of active oxygen formed by ultra-violet light. Stedman and Mendel [1926] have reported the presence of hydrogen peroxide in water and in protein solutions exposed to the rays of a mercury arc lamp. In the case of visible light on the other hand no photo-chemical action occurs in the absence of oxygen even when fluorescent dyes are present. In the presence of oxygen irradiation by visible light brings about a ready oxidation as Gaffron has shown. The selective action of ultra-violet light on tyrosine and tryptophan leads to the hypothesis that the action of visible light on living cells is essentially a photo-oxidation of certain specific constituents of the cell.

Method.

In determining the ability of organic compounds to undergo photo-oxidation the Barcroft technique for measuring oxygen uptake was employed. The flasks of the Barcroft apparatus were contained in a large water-bath at constant temperature, 20°. The source of light employed was a 300-watt 105-volt Osram lamp contained in a cylindrical glass vessel through which water continually circulated. The vessel was itself immersed in the water-bath at a distance of approximately 12 cm. from the flasks of the Barcroft apparatus. Under the conditions specified no light of shorter wave length than 320 $\mu\mu$ actually reached the contents of the flasks. Into each flask 2 cc. of the substrate were introduced. To one was added an appropriate volume of the sensitiser, to the other an equal volume of distilled water or buffer solution. Carbon dioxide evolved during the oxidation was absorbed by strong sodium hydroxide placed in a small container inside each flask. At the commencement of an experiment the flasks were shaken in the dark with taps open to

the air until temperature equilibrium was established. The taps were then closed and shaking continued in the dark. So long as light was rigorously excluded no oxygen uptake occurred, but on switching on the light measurable oxidation occurred within half a minute. The p_H of the solution was adjusted to 7·5.

The substances investigated were in the first instance purified by recrystallisation or distillation.

Chemical nature of substrate.

The problem of the relation of chemical structure to photo-dynamic activity which is raised by the work of Harris on amino-acids has been investigated by comparing the ability of a wide variety of organic compounds to undergo photo-dynamic oxidation. Differences of solubility and of the nature of solvent employed (water or alcohol) in different cases make quantitative comparison difficult and this has not been attempted. The aim of the present work has been to discover what types of compound undergo oxidation and what types are inactive. It is obviously outside the scope of the present work to attempt any comprehensive catalogue and certain types have been selected which throw light on the possibility of any particular chemical grouping having definite relation to photo-oxidation.

It may be stated in the first place that no exception has been discovered to the generalisation that the aliphatic compounds are inactive. Table I summarises the substances investigated in this series.

Table I.

Glucose	Sodium acetate	Acetone	Ethyl alcohol	Ethylamine
Fructose	Sodium lactate	Acetaldehyde	Aspartic acid	
Lactose	Sodium propionate			
Hexosediphosphate	Sodium oleate			
	Sodium linoleate			

It is evident that the presence of easily oxidisable groupings such as those present in oleic acid or acetaldehyde are not in themselves sufficient to ensure photo-oxidation. Nor does this inactivity depend on their ability or otherwise to absorb ultra-violet light. It is not until a complexity of structure represented by the aromatic compounds is reached that photo-oxidation is observed. Among this class of compound a wide variety of response is shown, and Table II summarises the results obtained.

Of the benzene derivatives studied in the above series it will be seen that the introduction of a hydroxyl or amino-group into the ring renders the compound susceptible to photo-oxidation. All the other groups studied—carboxyl, nitro-, cyano-, the halogens and sulphuric acid—are without effect. This conclusion is in harmony with the well-known fact that oxidation by methods other than that involving the influence of light is rendered easy by the introduction of amino- or hydroxyl-groups into the benzene ring as compared with

the refractory behaviour of the other groups mentioned. It will be seen that *p*-hydroxybenzoic acid gave a far greater oxygen uptake than any of the other substances investigated.

Table II.

Active.

Substance	O_2 mm.³/hour	Substance	O_2 mm.³/hour
o-Cresol	76·7	Tyrosine	425·0
m-Cresol	255·0	Benzaldehyde	360·0
p-Cresol	333·2	Phenylhydrazine	1025·0
Phenol	148·7	Cinnamic acid	55·0
p-Hydroxybenzoic acid	4500·0	Hypoxanthine	950·0
Aminobenzoic acid	525·0	Xanthine	2025·0
Salicylic acid	77·5	Uric acid	1000·0
Aniline	221·7	Guanine	900·0
α-Naphthol	320·0	Caffeine (p_H 7)	45·0
Tryptophan	1417·0	Caffeine (p_H 10)	170·0
Histidine	139·0		
Thiophen	300·0		
Quinoline	282·5		

Inactive.

Substrate	Substrate	Substrate
Benzene	Toluene	Anthracene
Benzoic acid	Benzyl alcohol	Quinine
Phthalic acid	Acetophenone	
Nitrobenzene	Hippuric acid	
Dimethylaniline	Nitrobenzaldehyde	
Cyanobenzene		
Iodobenzene		
Chlorobenzene		
Benzenesulphonic acid		

Turning now to the effect of introducing side-chains into the ring it will be seen that the presence of amino-groups such as those in tyrosine or phenylhydrazine or the occurrence of an unsaturated carbon as in cinnamic acid enables photo-oxidation to take place. It is of interest to compare phenylhydrazine and cinnamic acid on the one hand with hydrazine and oleic acid on the other. Although readily oxidisable groups are present in the latter pair photo-oxidation occurs only after introduction of the phenyl group.

The other side-chains investigated, as in benzyl alcohol, acetophenone, hippuric acid and toluene, are not oxidised. The only exception, benzaldehyde, is so unstable that oxidation is not surprising; and it was found that the introduction of a stabilising nitro-group (nitrobenzaldehyde) prevented oxidation.

The purine group, hypoxanthine, xanthine, uric acid, guanine and caffeine all showed a very large photo-oxidation. Owing to their insolubility they were investigated in alkaline solution at p_H 10. Caffeine in neutral solution was found to be nearly inactive. It is possible that the oxidation of nucleoproteins in the cell nucleus may be an important factor in the photo-oxidation of the living cell.

Dynamics of photo-oxidation.

The method adopted involved an examination of the rate of photo-oxidation of tyrosine in the manner already described under varying concentrations of substrate and sensitiser. In general the curve showing the rate of oxygen uptake closely resembled that obtained by Gaffron. The velocity of

the reaction is at first rapid, and in the case of higher concentrations of tyrosine nearly linear; but subsequently it becomes very slow and is almost at a standstill at the end of 9 hours. Fig. 1 shows the rates of oxidation of solutions of tyrosine at p_H 9 in a concentration of 200 mg. %, 100 mg. % and 50 mg. % respectively using 1 cc. of $M/2500$ solution of uroporphyrin as sensitiser.

At concentrations higher than 100 mg. % the velocity during the first hour is nearly linear and is independent of the initial concentration. The logarithm of the oxygen consumption when plotted against the duration of the reaction is in accordance with the view that the photo-oxidation of tyrosine is a monomolecular reaction following the law of mass action. The values

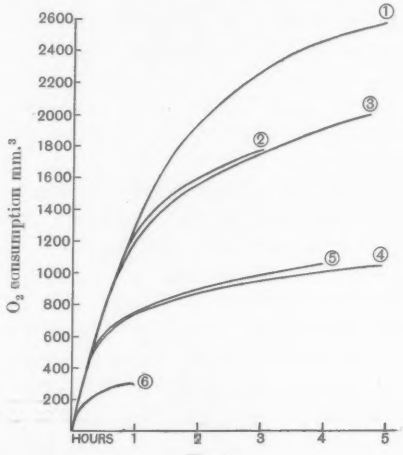

Fig. 1.

1. Tyrosine 200 mg. %.
2. Tyrosine 100 mg. %.
3. Tyrosine+glass powder.
4. Tyrosine 50 mg. %.
5. Tyrosine+glass powder.
6. Tyrosine 25 mg. %.

of k calculated at different stages of the reaction showed a gradual falling off. This is due in all probability to the gradual destruction of the catalyst used, namely porphyrin. The gradual change of colour of the reacting mixture during the course of the reaction from a pink to a reddish-brown, associated with a loss of fluorescence, is indicative of this change. The same effect is observed when methylene blue, eosin and haematoporphyrin are used as sensitisers. The nature of this slow destruction of the sensitiser is not clear but it appears to be an oxidative change. The porphyrins in the presence of oxygen and certain unknown impurities are slowly decomposed by daylight, especially in alkaline reaction, as shown by Squires, whereas eosin and methylene blue appear to be quite stable in the absence of a substance like tyrosine.

The interaction of substrate and sensitiser may possibly have some bearing on the problem of the relation of chemical structure of substrate to photo-

dynamic action. Harris found that the addition of small quantities of potassium cyanide had no influence on the velocity of the photo-chemical reaction in ultra-violet light, and this was confirmed in the case of visible light. It was found further that addition of small traces of ferrous or ferric iron to the system had no accelerating action, nor did the presence of these ions in the absence of a fluorescent dye bring about any photo-oxidation of tyrosine. This is hardly surprising since dilute solutions of these salts show only faint general absorption of light in the visible spectrum. It may be noted that Harris found that iron in ionisable form—particularly in the ferrous state—was able to act as an efficient light sensitiser, though less powerful in this respect than haematoporphyrin in ultra-violet light.

When varying concentrations of uroporphyrin were tried, the concentration of substrate remaining constant, it was found that the increased rate of oxidation at the higher level was not proportional to the increase in concentration of sensitiser. This is probably due to the fact that the surface layers absorb a large proportion of the incident light so that little oxidation occurs in the interior of the fluid.

The fact that the photo-chemical oxidation of tyrosine is of the monomolecular type suggested that the reaction might be a surface phenomenon analogous to that found for an enzyme action. It was thought of interest to see whether the rate of the reaction could be modified by increasing the available surface of the glass vessel by the addition of finely powdered glass. In order to remove grease and other impurities the powdered glass was boiled with nitric acid, washed in distilled water, alcohol and ether and finally dried. It was found that the addition of the powder to the flask containing tyrosine solution together with uroporphyrin or methylene blue produced no acceleration of oxidation. The curve of oxygen uptake could be superimposed on a curve obtained in the absence of glass powder. It was found, however, that above p_H 8·5 the autoxidation of uroporphyrin was very greatly accelerated, whereas this effect was negligible in the case of methylene blue.

In view of the importance of oxygen in the photo-dynamic effect of visible light in the presence of fluorescent dyes, experiments were made to test for the possible production of hydrogen peroxide during the course of the reaction.

It was found that whereas a preparation of catalase protected a culture of *Glaucoma* from the toxic effect of small concentrations of added hydrogen peroxide, no protective action was exhibited by catalase to the sensitisation to light of *Glaucoma* in the presence of haematoporphyrin. Tables III and IV show the results in the case of solutions of hydrogen peroxide and haematoporphyrin respectively.

On a number of occasions fluid from tubes containing *Glaucoma* culture and haematoporphyrin which had been exposed to light was tested by means of the guaiacum reaction, using freshly prepared alcoholic solution of guaiacum and an extract of horse-radish peroxidase. All these tests proved negative.

Table III.

Exp.	H_2O_2 0·077 %	H_2O_2 0·077 % catalase 1 drop	H_2O_2 0·077 % catalase 2 drops	H_2O_2 0·077% boiled catalase 2 drops	H_2O_2 0·73 % catalase 1 drop
O_9	19′	Motility reduced, 12 hours	Motility reduced, 12 hours	—	—
O_{10}	—	Not dead, 103′	—	25′	5′ O_2 evolved visibly
O_{11}	19′	Some dead. Remainder sluggish. 118′	—	—	5′

Table IV.

Exp.	*Glaucoma* 2 cc. Haematopor. 3 drops	*Glaucoma* 2 cc. Haematopor. 3 drops. Catalase 3 drops	*Glaucoma* 2 cc. Haematopor. 3 drops. Boiled catalase 3 drops	*Glaucoma* 2 cc. Haematopor. 3 drops Catalase 2 drops
YD	46′	—	—	74′
YE	66′	76′	76′	66′

It must be concluded that no hydrogen peroxide is formed during the course of a photo-chemical reaction in visible light. Some experiments were undertaken to see whether photo-dynamic action occurs in visible light when oxygen is replaced by a hydrogen acceptor. Methylene blue was chosen for this purpose since it can act both as hydrogen acceptor and as fluorescent sensitiser. Solutions of tyrosine together with a small amount of methylene blue were placed in Thunberg tubes which were then evacuated and exposed to visible light for periods up to 24 hours. Decoloration of methylene blue took place slowly and was nearly complete at the end of this time. Control tubes containing methylene blue and tyrosine kept in the dark after evacuation showed no trace of decoloration. It was found possible to replace tyrosine by other substances such as phenol or guanine which undergo photo-oxidation in the presence of oxygen. Sodium nitrite, which gives no oxygen uptake under aerobic conditions, failed to decolorise methylene blue anaerobically when exposed to light. Replacement of methylene blue by a sensitiser like haematoporphyrin, which does not act as a hydrogen acceptor, does not bring about the photo-oxidation of tyrosine under anaerobic conditions.

Summary.

1. The relation of chemical structure to the phenomenon of photo-dynamic action has been investigated. None of the aliphatic compounds employed was found to undergo photo-oxidation. Of the benzene derivatives only those in which an amino- or hydroxyl group has been introduced into the ring were oxidised. Side chains in which the groups $-NH_2$ and $-CH=CH-$ occur are oxidised. The other side chain compounds investigated are inactive. Certain other ring compounds, α-naphthol, tryptophan, thiophen and, to a marked degree, the purine derivatives, are oxidised.

2. The significance of this selective action in relation to the photo-oxidation in the living cell is pointed out.

3. The photo-oxidation of tyrosine corresponds to a monomolecular reaction following the law of mass action.

4. During the course of the reaction the photo-catalyst is gradually destroyed. This is true of uro- and haemato-porphyrin and of eosin and methylene blue. The last two do not undergo autoxidation as in the case of the porphyrins.

5. The presence of ionised iron salts and of glass powder has no accelerating influence on the reaction. Potassium cyanide does not inhibit the reaction. The behaviour of iron salts in visible light contrasts with their activity as sensitisers in ultra-violet light.

6. Hydrogen peroxide is not formed during the course of the reaction.

7. Methylene blue brings about the oxidation of tyrosine and other substances under anaerobic conditions in the presence of visible light. It appears to act in this respect both as photo-catalyst and hydrogen acceptor.

In conclusion I wish to express my sincere thanks to Prof. R. A. Peters for his continual help and encouragement, and to Mr Whitley for a generous grant held during the course of this work.

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LXXIV. BIOCHEMISTRY OF PLANT DISEASES.

X. FERMENTATION OF PENTOSES BY *FUSARIUM LINI*[1].

By MOLLIE GRACE WHITE and JOHN JAMES WILLAMAN.

From the Division of Agricultural Biochemistry, University of Minnesota.

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A series of studies has been in progress in this laboratory for several years on the metabolism of *Fusarium lini* Bolley, the causal organism of flax wilt. Tochinai [1921, 1, 2] first pointed out the omnivorous nature of the organism, as regards both carbohydrates and non-carbohydrates. He also noted the production of carbon dioxide in fermentation tubes. This suggested that ethyl alcohol might also be produced, and led to the work reported in the first papers in this series on *F. lini* [Anderson and Willaman, 1922; Anderson, 1924; Letcher and Willaman, 1926].

Since the investigations reported in the present paper are a sequel to Anderson's results, and since his paper is not very generally available, a brief review of his results is given. He attempted to account for the carbon added to the medium in the form of glucose and of xylose. Ethyl alcohol was found in considerable quantities. In fact it accumulated to the extent of 4·33 % in some media. On a glucose medium, carbon dioxide, alcohol, and mycelium accounted for from 90 to 95 % of the carbon consumed. Furthermore, the ratio of carbon dioxide to alcohol during the early period of the fermentation was very nearly the same as is required by the conventional equation of alcoholic fermentation by yeast:

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH.$$

The rate of fermentation was far slower than that by yeast, maximum alcohol content being attained between the 12th and 20th days. After reaching this maximum the alcohol content always decreased, suggesting its consumption by the fungus. This was proved by placing the fungus in a medium containing only alcohol as a source of carbon. Growth was immediate and fairly rapid, but was inhibited altogether if the original alcohol content was above 3·5 %.

On a xylose medium a somewhat different picture of metabolism was

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obtained. The same products were noted, but the ratio of carbon dioxide to alcohol was such as to require the following general reaction:

$$C_5H_{10}O_5 \rightarrow 2CO_2 + C_2H_5OH.$$

It will be noted that this reaction does not account for one carbon and four hydrogen atoms. Furthermore, the total carbon recovered in the various products in the culture was only about 80 %.

The present investigations were undertaken (1) to study more carefully the metabolism of *F. lini* on the pentoses, and (2) to examine still further the obvious similarity between the metabolism of this fungus and that of ordinary yeast, especially as regards the intermediate products of alcoholic fermentation. The first phase of the work is presented in the present paper and the second in the following paper [White and Willaman, 1928].

Search for urea as a product of metabolism.

Since, as indicated above, one carbon and four hydrogen atoms are required to complete the equation of fermentation of *F. lini* on xylose, it seemed reasonable to suppose that urea might be the unidentified substance. The literature contains numerous references to the occurrence of urea not only in higher plants but in fungi [Bamberger and Landsiedl, 1903; Fearon, 1926; Gaze, 1905; Iwanoff, 1923, 1924, 1925]. Fosse's methods for both cyanates and urea, involving the use of xanthhydrol [Fosse, 1907, 1913], were applied to a large number of cultures of various ages on xylose, arabinose, rhamnose and glucose. Some indication of urea was obtained on the last medium, but on none of the others was there any convincing evidence of either urea or cyanates. Anderson found that urea serves *F. lini* excellently as a source of nitrogen. Hence, if urea were formed by the fungus it might be reabsorbed and utilised, just as is the case with ethyl alcohol.

Products of metabolism on pentoses.

Since it appeared very unlikely that urea could account for the low total carbon recovered by Anderson in the xylose media, it was decided to make a more careful study of the known products of metabolism, in the hope that more refined methods of analysis and of handling the cultures might explain the discrepancies.

Form 17 of *F. lini* [Broadfoot, 1926], furnished by the Division of Plant Pathology of the University of Minnesota, was grown on 4 % nutrient solutions of xylose, arabinose, and rhamnose. At intervals the cultures were analysed for mycelium, CO_2, ethyl alcohol, unchanged sugar, and lead precipitate. The latter represents a very small quantity of some unknown organic material. The results are given in Figs. 1, 2, and 3, in terms of carbon. The complete data are in Tables I and II.

On xylose the total recovery of the carbon approximates to 95 %, with the exception of the analyses on the 10th day, when it is 80 %, and the 40th day,

when it is 97 %. The recovery on the 10th day is evidently due to an error in the alcohol determination. From the gradual increase of the percentage of carbon as alcohol in the rest of the series we are inclined to think that alcohol production was normal throughout the 40-day period on the xylose medium. The trend of the curves is very similar to that of those obtained by Anderson, except that he accounted for only 80 % of the carbon. On arabinose the accounting for the total carbon among the products of metabolism is much more nearly a straight line, and averages 99 %. In the early analyses, where the quantities of products were small and their determination consequently more difficult, the total carbon accounted for is 99·5 %, which is a much better result

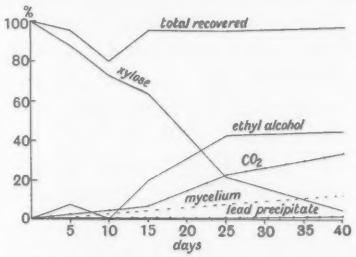

Fig. 1. Distribution of carbon among the products of metabolism on xylose.

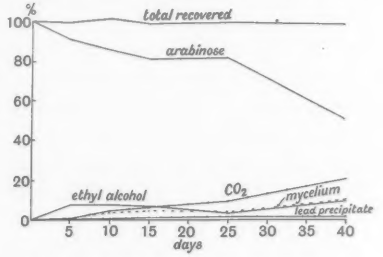

Fig 2. Distribution of carbon among the products of metabolism on arabinose.

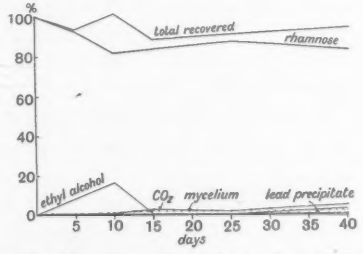

Fig. 3. Distribution of carbon among the products of metabolism on rhamnose.

than was obtained with either xylose or rhamnose. The average of the total return of carbon on rhamnose is about 94 %, more nearly like xylose than arabinose.

From the results of the totals obtained in this series of studies on three pentoses it seems doubtful if there is a product unaccounted for. Rather it would appear that the method of making all of the analyses for carbon on one culture (as described in the experimental part), instead of on a set of three cultures as Anderson did, makes for more exactness. No method yet described for quantitative determination of small amounts of ethyl alcohol gives one a feeling of satisfaction. With more attention to detailed accuracy in the estimation of the products in these metabolism studies, it is quite probable

that there would be no discrepancy between the carbon consumed and the carbon accounted for among the products already recognised.

The total amount of sugar consumed and the rate of consumption are noticeably different in the three cases and, using as a criterion the quantities consumed, one may infer that the suitability of xylose, arabinose, and rhamnose as sources of carbon for this fungus decreases in the order given. The rate of consumption of the xylose seemed somewhat higher here than in Anderson's studies, since he found that the xylose was not entirely utilised until the 56th day. The daily utilisation of xylose was roughly 2·4 % of the original sugar, compared to 1·8 % in his investigation. He used Form 10 of the fungus, whereas in the present work it was Form 17. This may easily explain the variation. The rate of utilisation of arabinose shows a gradual decrease from 1·9 % daily to 0·7 % and then increases to 1·1 % during the last growth period. The rate of utilisation of the rhamnose is at a maximum at 10 days, rising from 1·5 % on the 5th day to 1·7 % on the 10th day, after which there is a sharp drop on the 15th day to 1 % and to less than 0·4 % on the 40th day. The values calculated from the average found in the different growth periods give a rough estimate of the rate of utilisation of the three pentoses as 100 : 47 : 38 for xylose, arabinose, and rhamnose, respectively.

One of the most obvious comparisons in these curves is that between the alcohol found in the medium and the sugar consumed. In all three charts these pairs of curves are shaped as rough mirror images of each other. When sugar consumption is rapid, more alcohol is found. Anderson found that on glucose the alcohol content attained a maximum and then decreased, supposedly because it was consumed by the fungus. In the case of rhamnose, our interpretation is that rhamnose is utilised with such difficulty that as soon as appreciable quantities of alcohol are present the fungus uses the alcohol instead. The same tendency, although in less marked degree, can be seen in the arabinose curves.

Alcohol accounted for a greater percentage of the carbon, and was produced in greater amount, on the xylose medium than on either of the others. On xylose, except for the unfortunate low determination on the 10th day, the percentage of the carbon in the alcohol fraction increased from 7·0 % on the 5th day to 44·5 % on the 40th day. The quantity of sugar consumed in the same time was approximately 16·7 % of the original amount, showing that in this case there is apparently no indication that the fungus could utilise the alcohol for its source of carbon more easily than it could use the sugar.

Carbon dioxide production showed a regular rise in all three media, though the quantity produced in each was decidedly different. At 40 days its ratio in the xylose, arabinose, and rhamnose media was 100 : 60 : 15. The relationship between the carbon dioxide and alcohol was noted with some interest in this study, since the ratio of carbon as alcohol to carbon as carbon dioxide was thought by Anderson to be 1 : 1 on xylose as contrasted with 2 : 1 on glucose. On the 15th day on xylose the ratio was 2·6 : 1; on the 25th day,

1·86 : 1; on the 40th day, 1·30 : 1. For arabinose and rhamnose the ratio is very high for the first 10 days and then drops below 0·5 on the 40th day From these results it is quite apparent that no simple fermentation equation may be formulated, for the trend of events changes continuously, due largely, it is believed, to consumption of alcohol by the fungus.

Much of the judgment of suitability of various compounds as a source of carbon or as a source of nitrogen for an organism is based upon the dry weight of mycelium produced in a given time. Using this criterion among the three pentoses studied it is evident that arabinose is only slightly less favourable as a source of carbon than xylose, whereas rhamnose is apparently only about one-fourth as suitable as xylose. This same conclusion must be drawn no matter what set of products is compared, and it is natural to ascribe the disparity to the presence of the methyl radical in the rhamnose molecule, although no direct evidence for this conclusion is as yet available.

Anderson found some evidence for the presence of succinic acid in cultures of *F. lini*. He therefore always included in his analysis a treatment of the medium by lead acetate. This was also done in the present work. It is listed simply as the carbon in the lead precipitate, and no further attempt has been made to identify it. It never amounts to more than 1·6 % of the total carbon, although it increases steadily with the age of the cultures.

Peterson, Fred and Schmidt [1922] adopted a series of coefficients for studying the ratios among the products of fungus metabolism. These coefficients have been calculated for the present data, and are shown graphically in Fig. 4. Perhaps the most outstanding feature of the graph is the peak to which the economic coefficient rises by the 5th day, and its immediate drop on the 10th or 15th day to a level which is maintained during the rest of the life of the culture. This indicates that during the early periods, although the sugar is consumed, it is not effectively utilised for growth, but for alcohol production. This is borne out by the shape of the alcohol curves. The respiration coefficient, which indicates the relation between CO_2 production and growth, shows a gradual increase, and has about the same value for all sugars. The respiration equivalent, which is the percentage of the carbon consumed that is transformed into carbon dioxide, has a distinctly different value for the three sugars studied. In the case of xylose the average value is not far from 25; with arabinose the value is much nearer 50; and in the case of rhamnose the average figure is about 16. The plastic equivalent, defined as the percentage of carbon consumed which is used to build mycelium, again has the highest value for arabinose. Rhamnose and xylose are not far apart but it is noticeable that again rhamnose has a very gradual order of ascending values, while xylose shows its highest values on the 10th and 15th days and then a decrease to the 25th and 40th days.

From these curves we may conclude that arabinose is the most economical of these three sources of carbon for the building of mycelium, with rhamnose approximately half as economical after the 10th day, and xylose the least of

the three. In other words the fungus produces the most alcohol on the sugar on which it produces the least mycelium, namely xylose. This fact might become of importance in any attempt to utilise wood waste for alcohol production by means of this fungus.

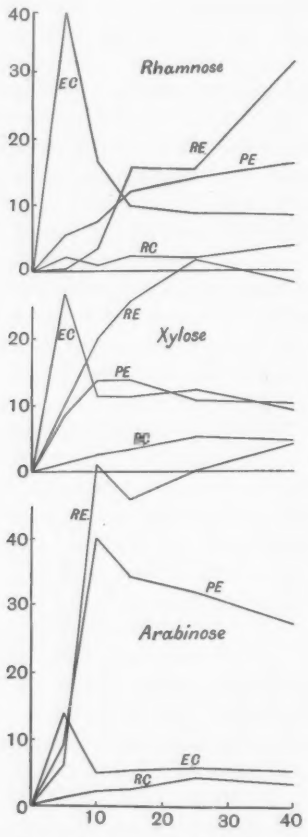

Fig. 4. Various ratios among the products of metabolism on xylose, arabinose, and rhamnose.

$$RC = \text{Respiration coefficient} = \frac{\text{Weight of } CO_2}{\text{Weight of mycelium}}.$$

$$EC = \text{Economic coefficient} = \frac{\text{Weight of sugar consumed}}{\text{Weight of mycelium}}.$$

$$RE = \text{Respiration equivalent} = \frac{\text{Carbon of } CO_2}{\text{Carbon consumed}} \times 100.$$

$$PE = \text{Plastic equivalent} = \frac{\text{Carbon of mycelium}}{\text{Carbon consumed}} \times 100.$$

Experimental details.

Preparation of sugars.

The pentoses used in these experiments were prepared by the writers, using especial care to obtain sugars of the standards of purity described by Pfanstiehl and Black [1921].

Cultures.

Cultures of *Fusarium lini* were kindly furnished for this research by Dr Louise Dosdall, mycologist for the division of Plant Pathology of the University of Minnesota.

All media in which the sugars were used as a source of carbon for the organism were made from a stock solution of the following composition: $MgSO_4,7H_2O$, 0·25 g.; KH_2PO_4, 0·50 g.; NH_4NO_3, 1·00 g.; water to make 1 litre. The sugars were added to the above medium to the extent of 4 g. per 100 cc. Seven 500 cc. conical flasks were prepared for each series, each flask containing 200 cc. Two of the flasks were closed with standard hospital cotton plugs, and the remaining five were supplied with two-holed rubber stoppers carrying a right-angled glass entrance tube and an exit tube which nearly reached to the liquid in the flask. The outer extremity of these tubes was plugged with cotton and all flasks were sterilised in the autoclave for 20 minutes at 15 pounds' pressure. Inoculation was made by means of a platinum needle, introducing as nearly as possible the same amount of mycelium in each case. The glass tubes were then closed with rubber tubing, fastened firmly with screw clamps. The seven flasks were incubated at $28° \pm 1°$. The five flasks provided with rubber stoppers were for analysis at five subsequent periods of growth. The two extra flasks were provided so that a determination, other than for carbon dioxide or ethyl alcohol vapours, could be duplicated on a culture of the same age as one lost in case of accident.

Once a day the flasks were removed from the incubator and CO_2-free air was drawn through the flask for 10 minutes while the flask was in the respiration train. The latter consisted of a bead tower of baryta, the flask itself, a bead tower containing 25 cc. of concentrated sulphuric acid for collecting the alcohol vapours, and a bead tower containing 50 cc. of normal NaOH.

Analytical methods.

When the contents of a flask were analysed, the vapours were drawn off first, then the mycelium was carefully filtered and washed on a tared filter paper and dried. The filtrate from the culture and the wash waters were put together and made up to a definite volume, and on aliquot parts of this solution determinations were made for unused sugar, ethyl alcohol, lead precipitate, urea, and cyanate.

The *unused sugar* was determined by the method of Willaman and Davison [1924], which is a modification of the picric acid method, and these determinations were checked by the iodine method as used by Cajori [1922].

Tests were made for *urea* and *cyanates* by the method of Fosse [1907, 1921]. He detects and isolates urea as the insoluble condensation product which it forms with xanthhydrol. Urea in dilutions up to 1 in 20,000 may be detected by the proper use of this reagent. Cyanates are often present in the same solution with urea and since they readily form urea by tautomerism on heating in the presence of ammonia, it is possible to test the same solution for both substances with the same reagent.

The *lead precipitate* was formed by adding an excess of 10 % normal lead acetate, filtering, washing, drying; the carbon was determined by the method cited below.

The *alcohol* content of the filtrate was determined densimetrically, using both a specific gravity balance and a pyknometer. The alcohol in the vapours, collected in the towers of the respiration train, was estimated separately by the method of Dox and Lamb [1916] by oxidation to acetic acid, distillation and titration of the latter.

The *carbon dioxide* absorbed in the tower of *N* NaOH was found by emptying the beads into the flask carrying the tower and titrating after the addition of barium chloride.

Table I. *Products of metabolism of* Fusarium lini *when grown on* 200 *cc. of medium containing* 8·00 *g. of the different pentoses.*

Pentose	Age of culture days	Mycelium			CO_2	Alcohol	Carbon in lead precipitate	Sugar
		Weight g.	Carbon %	Nitrogen %	g	g.	g.	g.
Xylose	5	0·036	43·0	5·9	—	0·43	0·013	7·04
	10	0·192	48·0	6·0	0·48	0·01	—	5·82
	15	0·261	50·7	5·8	0·90	1·20	—	5·10
	25	0·522	46·7	5·2	2·68	2·59	0·019	1·73
	40	0·837	46·6	5·8	3·98	2·70	0·050	0·39
Arabinose	5	0·055	49·4	6·3	0·07	0·48	—	7·25
	10	0·239	45·8	6·2	0·51	0·48	0·019	6·82
	15	0·298	49·2	5·7	0·72	0·40	0·034	6·44
	25	0·272	47·2	4·1	1·11	0·20	0·048	6·49
	40	0·690	48·1	3·4	2·44	0·51	0·055	4·44
Rhamnose hydrate	5	0·015	82·6	9·0	0·03	0·00	0·003	7·42
	10	0·089	47·6	6·5	0·07	1·03	0·007	6·55
	15	0·134	46·3	5·7	0·29	0·10	0·010	6·69
	25	0·117	47·8	5·8	0·23	0·00	0·006	6·99
	40	0·163	51·8	5·6	0·61	0·20	0·012	6·66

The *carbon* content of the mycelium and of the lead precipitate was determined by a modification of the wet combustion method employed by Gortner [1916]. Instead of weighing the carbon dioxide in potash bulbs, it was absorbed in approximately 0·2 *N* NaOH solution in a cylindrical flask containing a coiled glass tube [Beaumont *et al.*, 1928], and titrated after the addition of barium chloride.

The method suggested by Anderson and Schutte [1924] for the determination of the *nitrogen* of the mycelium from the residue left from the carbon determination was found to be highly satisfactory.

Table II. *Percentage distribution of carbon among the products of metabolism of* Fusarium lini *on various pentoses.*

Pentose	Age of culture days	Proportion of the original carbon in: Mycelium %	CO_2 %	Alcohol %	Lead precipitate %	Sugar %	Total %
Xylose	5	0·5	—	7·2	0·4	88·0	96·1
	10	2·9	4·1	0·1	—	72·7	79·8
	15	4·1	7·6	19·8	—	63·7	95·2
	25	7·6	22·8	42·6	0·6	21·6	95·2
	40	12·2	33·9	44·5	1·6	4·9	97·1
Arabinose	5	0·8	0·6	7·6	—	90·6	99·6
	10	3·4	4·4	7·6	0·6	85·2	101·2
	15	4·6	6·1	6·6	1·0	80·4	98·7
	25	4·0	9·4	3·3	1·5	81·2	99·4
	40	10·4	20·8	9·9	1·7	55·5	98·3
Rhamnose	5	0·4	0·3	0·0	0·1	92·7	93·5
	10	1·3	0·6	16·7	0·2	81·9	100·7
	15	2·0	2·5	1·6	0·3	83·6	90·0
	25	1·8	1·9	0·0	0·2	87·3	91·2
	40	2·7	5·3	3·3	0·4	83·2	94·9

SUMMARY.

When *Fusarium lini* is grown on xylose, arabinose, or rhamnose, the main products of metabolism are mycelium, CO_2, ethyl alcohol, and a small amount of organic substance precipitated by lead. From 95 to 99 % of the carbon can be accounted for in these products, making it rather unlikely that there is any other product of major importance.

Of these three pentoses, xylose is utilised the most efficiently for alcohol production and arabinose for growth. Rhamnose is utilised very poorly for either.

During the course of growth in a culture the ratio of CO_2 to ethyl alcohol is constantly shifting, due to the consumption of alcohol by the fungus.

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LXXV. BIOCHEMISTRY OF PLANT DISEASES.

XI. *FUSARIUM LINI* AND THE PYRUVIC ACID THEORY OF ALCOHOLIC FERMENTATION[1].

By MOLLIE GRACE WHITE and JOHN JAMES WILLAMAN.

From the Division of Agricultural Biochemistry, University of Minnesota.

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Previous papers in this series [Anderson, 1924; White and Willaman, 1928] have brought out the facts that the principal products of metabolism of *Fusarium lini* Bolley, when grown on hexoses or pentoses, are carbon dioxide and ethyl alcohol, and that these two products, together with the mycelium produced, account for over 95 % of the sugar consumed. Certain comparisons with yeast fermentation are suggestive: the fungus and the yeast are alike in the ratio of CO_2 to alcohol produced on a hexose medium, but they differ in that ordinary yeast utilises the pentoses poorly if at all, that the fungus consumes the alcohol which it has produced, and that the rate of fermentation by the fungus is a matter of days instead of hours. Because, however, of the obvious similarity in end-products, it was thought desirable to examine the analogy still further. At the present time the pyruvic acid scheme of yeast fermentation is rather generally accepted. Since excellent reviews of this subject have lately appeared [Harden, 1923; Nord, 1926], it will be sufficient here simply to outline the supposed chain of events. The hexose molecule first is split into two three-carbon chains; methylglyoxal is the most reasonable of such three-carbon molecules that has been suggested, and it is the one usually adopted in the theory under discussion. Two molecules of this substance then undergo a "biological Cannizzaro" reaction, producing glycerol and pyruvic acid. The latter is decarboxylated to acetaldehyde which, with another molecule of methylglyoxal, then undergoes a mixed Cannizzaro reaction, with the formation of ethyl alcohol and of more pyruvic acid.

Although a great deal of work has been done to demonstrate this scheme in the case of yeast, only partial success has resulted. The proof would have to consist in showing that if any of the alleged intermediates are fed to the yeast the latter will utilise them for the production of alcohol and CO_2, and continue the course of events demanded by the scheme.

The present paper is a report of experiments in which the above intermediates were fed to *F. lini*, besides a number of other similar compounds.

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Methylglyoxal itself has proved to be too reactive a compound to be dealt with in cultures; therefore its hydroxy-derivative has been used instead. Glycerol has been shown by Tochinai [1921] to be readily used by the fungus, hence it was omitted here. Pyruvic acid and acetaldehyde are the other intermediates. Besides these, acetone, dihydroxyacetone, furfuraldehyde, oxalic and citric acids, and cinnamic aldehyde were used.

Growth on hydroxymethylglyoxal was immediate and fairly vigorous. Attempts were not made to measure the CO_2 and alcohol produced. Methylglyoxal itself was found by Mayer [1907] and by Wohl [1907] to be unfermentable by yeast. Buchner and Meisenheimer [1906] obtained the same results using both acetone-yeast and yeast juice. The products of fermentation would probably not be the same from the hydroxy-compound as from methylglyoxal, but such products have not as yet been looked for.

Growth on pyruvic acid was slower and less abundant than on the above, but production of CO_2 was very rapid. At the end of 12 days, 10 % of the original carbon was obtained in this form. Apparently the fungus can decarboxylate the acid very readily. Ethyl alcohol could not be detected. Since alcohol has been shown to be readily utilised by the fungus [Anderson, 1924], it is possible that, as fast as the pyruvic acid is decarboxylated to acetaldehyde, the latter is converted to alcohol and used by the fungus for growth. This view is supported by the fact that in an acetaldehyde culture growth readily takes place, with the production of only traces of CO_2 but of sufficient alcohol to be readily detected by the odour.

Acetone was tolerated in concentrations up to 10 %. Growth was immediate and rapid, but only small amounts of CO_2 were produced. No alcohol could be detected.

Dihydroxyacetone proved to be readily available for the fungus; a heavy mat of mycelium was produced, and CO_2 was formed in amount comparable to that on the pentoses.

We may now compare *F. lini* with yeast as regards the extent to which they have been demonstrated to fit into the pyruvic acid theory. Methylglyoxal has not been tried on the fungus; it is not utilised by yeast. Its hydroxy-derivative is not used by yeast [Nord, 1927], but it is by the fungus. Glycerol is utilised only by the fungus. Pyruvic acid is utilised by both, but much more readily for growth by *F. lini*[1]. Acetaldehyde is available for both, and is detectable in the medium when both organisms are grown on hexoses [Letcher and Willaman, 1926]. Ethyl alcohol is produced by both, and is readily utilised by the fungus for growth, but not by the yeast.

Thus it has been demonstrated that each intermediate compound in the pyruvic acid theory of alcoholic fermentation may be utilised by *F. lini*, the methylglyoxal being represented by its hydroxy-derivative. Although ethyl

[1] Dr F. F. Nord was present when these experiments were conducted, and made some of the comparisons with yeast. The writers are also indebted to him for many suggestions during the course of this work, and for assistance in preparing the hydroxymethylglyoxal.

alcohol has not been found in all cultures, it is probably produced and then consumed by the fungus in preference to the original compound. Although these results do not constitute proof of the pyruvic acid theory for this fungus, nevertheless there seems to be a rather close analogy between the mechanism of alcohol fermentation in yeast and in *F. lini*.

Experimental.

Preparation of substances.

The *pyruvic acid* was purified by distilling it, at a pressure of 8 mm. at 55°, in a stream of CO_2.

The *cinnamic aldehyde* was distilled at a pressure of 10 mm. at 120°.

The *acetaldehyde* was not purified, but transferred to the sterile culture solution immediately after breaking the seal of the container.

The *hydroxymethylglyoxal* was prepared from dihydroxyacetone by the method of Evans and Waring [1926].

The *furfuraldehyde* was distilled at about 3 mm. pressure, the middle fraction of the distillate being used.

The acetone, dihydroxyacetone, and citric and oxalic acids were used as secured from the manufacturers.

Cultures.

The mineral nutrient solution used as a medium had the following composition: $MgSO_4,7H_2O$, 0·25 g.; KH_2PO_4, 0·25 g.; NH_4NO_3, 1·0 g.; water to make 1 litre.

100 cc. of this medium in a 300 cc. conical flask were sterilised, and then the organic compound was added. This was necessary because of the volatile nature of most of the substances.

Incubation was at 28°.

Alcohol and CO_2 were determined on some of the cultures. Since alcohol was found only in the acetaldehyde culture, and since the results here may have been vitiated by the volatility of the aldehyde, no alcohol data are presented.

The results are given in Tables I and II.

Table I. *Results of cultural studies on non-sugar media.*

Compound	No. of cultures	Range of concs. %		Conc. for best growth %	Time for noticeable growth days	Time for max. growth days	Type of final growth
Pyruvic acid	12	0·05 to	0·1	0·1	3	18	Fine mycelium
Acetaldehyde	16	0·01	0·2	0·1	2	9	„ „
Acetone	16	0·01	10·0	10·0	0·5	21	Heavy clumps
Cinnamic aldehyde	6	0·25	1·0	No growth	No growth	No growth	
Citric acid	8	0·05	2·0	2·0	1	21	Heavy mat
Dihydroxyacetone	8	1·00	2·0	2·0	2	12	„ „
Hydroxymethylglyoxal	6	0·01	0·1	—	1	12	Clumps of mycelium
Oxalic acid	4	0·05	0·1	—	3	10	Fine mycelium
Furfuraldehyde	4	0·10	0·5	0·1	1	—	„ „

Table II. *Production of carbon dioxide by* Fusarium lini *on various non-sugar compounds.*

Compound	Conc. %	Carbon dioxide at the end of 3 days mg.	6 days mg.	9 days mg.	12 days mg.
Acetaldehyde	0·2	18·2	21·6	36·5	27·3
Acetone	10·0	12·9	21·6	30·8	22·8
Pyruvic acid	0·1	23·7	34·2	59·4	75·4
Dihydroxyacetone	2·0	20·9	32·9	29·6	59·4

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LXXVI. STUDIES ON PECTIN. PART II.

THE ESTIMATION OF THE INDIVIDUAL PECTIC SUBSTANCES IN NATURE.

By DINSHAW RATTONJI NANJI
and ARTHUR GEOFFREY NORMAN.

From the Department of Biochemistry, University of Birmingham.

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Introduction.

The object of the present work was to study the distribution of the individual pectic substances in nature. Whereas the total pectic content of such materials as apples, exhausted beet slices, etc., is well known, since they are of commercial value by reason of their relatively high content of pectic substances, no determinations have as yet been published on the distribution of these substances generally in the vegetable kingdom. An attempt in this direction was commenced by Hornby [1920] on fruits and roots, but the method of estimation adopted by him is open to criticism as will be indicated later.

In addition to determining the total and regional contents of the material under investigation, a separation into the various types of pectic substance has been effected by differential extraction. Histological investigations have been carried out on similar lines, and these will be published elsewhere.

In the preliminary work involved, which need not be described here, two main difficulties had to be overcome. First, a method of extraction which was quantitative and yet suitable for routine work had to be devised, and secondly, it was necessary to elaborate an accurate method of estimation. The principle involved in the estimation is that put forward by Carré and Haynes [1922]. In brief, it consists in precipitating the pectin as calcium pectate, after hydrolysis by alkali, and weighing as such. Although rather involved, the method, with due precautions, is accurate.

It is proposed eventually to follow by this method the regional and seasonal changes in some selected types of plant or plants, at the same time proceeding with histological investigations on the same material.

Extraction.

Carré and Haynes [1922] first removed the soluble pectin by extraction with cold water, and then the form insoluble in water by another method. It is obvious that any incompleteness in this initial extraction would render

the later results unreliable. In addition a cold water extract would in all probability remove little more than the pectin in solution in the cell sap. The method they adopt is to freeze the material for some hours to ensure that it is completely killed, after which it is brought to the ordinary temperature and pressed through cloth with the solvent in a hand press. The residue is ground with sand, washed with the extracting liquid and pressed. Sixty to eighty extractions were usually required. So laborious a method is obviously unsuitable for a large series of estimations. In addition it is doubtful whether such repeated extraction with large volumes of solvent is sound in principle, as it is conceivable that an insoluble colloidal substance, such as pectic acid, might become dispersed, and passing into apparent solution vitiate the results.

Carré [1922] estimated the insoluble pectin or protopectin by heating the residue after extraction of the water-soluble pectin with $N/10$ HCl in an autoclave for 1 hour at a temperature of 110°. The protopectin, she states, is converted into the soluble form "presumably by hydrolysis." Such a method, however, cannot be considered accurate, as acid treatment in the autoclave produces very considerable hydrolysis of the pectin itself beyond the pectic acid stage.

Tutin [1925], for the estimation of pectin in certain pathological leaves, first cut them into strips and immersed them in boiling alcohol. After several treatments with alcohol they were dried and weighed. The pectin was then extracted with boiling water.

It is evident that the chief difficulty to be met with is that of obtaining uniformly complete extraction. The difficulty is purely a structural, not a chemical, one, *i.e.* the inability of the extracting agent to penetrate properly into all the cells of the tissue, and its failure to reach and dissolve out those insoluble pectic substances intimately connected with the cell-wall, either as an incrusting substance or in the form of the middle lamella. Thus it would appear that for complete extraction the material in question must be in a very fine state of division. Link and Tottingham [1923] have studied the effects of various methods of desiccation of plant materials, and they come to the conclusion that drying at 95° is satisfactory if the starch or sugar content of the tissue is not too high. This method accordingly was adopted for all tissues. In the case of one or two fruits the sugar appeared to caramelise to a certain extent, but there is reason to believe that very little error is caused through this.

The materials were taken in as fresh a condition as possible and placed in thin layers in a hot steam-oven, so that the enzymes were destroyed almost immediately. Seeds were first ground up, and fruits cut into thin slices. Moisture estimations were made. When completely dry the materials were ground as finely as possible, sieved in graded sieves, and re-ground to obtain a uniformly fine powder, which was used for the extractions.

The optimum time and temperature of extraction had next to be determined. It is clear that a mean period had to be found at which extraction would be complete, yet not so prolonged that hydrolysis might take place

beyond the pectic acid stage. For this reason actual boiling was avoided, the flasks being placed on a copper water-bath about 2 inches above the level of the water, the temperature of the extracting liquid being then 85°. The following table represents the yield of calcium pectate obtained per 100 g. of dried sycamore leaf powder by extraction with water at 85° for various periods.

Table I. *Extraction of sycamore leaf powder with water.*

Time of extraction in hours	Percentage yield of calcium pectate
6	1·42
12	2·30
18	3·30
24	3·72
36	3·70

As a result of this and many similar experiments, extraction was considered complete at the end of 24 hours. The residue after washing was re-extracted with water for a considerable period, but no further trace of pectin could be found.

A comparison was made using the above method of extraction for 24 hours on the same leaves taken fresh but ground with sand and water very finely. The calcium pectate yield per 100 g. calculated on the dry weight was only 0·9 in this case, compared with 3·7 on the powdered leaf.

The solutions used in addition to water for extraction in the routine investigations were oxalic acid and ammonium oxalate. It was indicated by Norris and Schryver [1925] that the best concentration both of oxalic acid and of ammonium oxalate for the extraction of pectic substances is 0·5 %. This observation was confirmed and this concentration only employed in each case.

Estimation.

The earliest method of estimation adopted was that indicated by von Fellenberg [1918] in which the pectin is precipitated by alcohol and weighed. It is obvious that this method is inaccurate, inasmuch as any other substances in the extract precipitable by alcohol would be weighed as pectin. In addition, pectin, on precipitation, almost invariably carries down with it considerable quantities of any other substances present in the solution, which are very difficult to wash out subsequently. Also, as the dilution of the pectin increases, precipitation becomes increasingly difficult. Nevertheless, this method was advocated by Wichmann [1922, 1923]. Hardy [1924] has given figures for both calcium pectate yield and alcohol precipitate of the same extract. In every case the alcohol precipitate was found to be much higher owing to the presence of impurities. Hornby [1920] employed a method of estimation suggested by the work of von Fellenberg [1918]. In this case the methoxyl yield of the tissue is determined directly, and pectin obtained by calculation on the von Fellenberg formula. This presupposes pectin in all natural sources to have the same methoxyl content, and further, assumes that pectin is the sole source of methoxyl groups in vegetable tissues.

The calcium pectate method of estimation referred to previously was described by Carré and Haynes [1922]. Briefly it consists of adding to the pectin solution $N/10$ sodium hydroxide to convert the pectin to the soluble sodium pectate. After acidification with acetic acid, calcium chloride is added, and the pectate deposited as the insoluble calcium salt. They state that acetic acid in excess removes practically all impurities. Tutin [1923] criticises this on the grounds that any acid yielding a calcium salt insoluble in acetic acid would be weighed as pectin. It is obvious that this method would be impossible using oxalic acid or ammonium oxalate as the extracting agent.

The method finally adopted involves precipitation by alcohol, re-solution of the precipitate, and subsequent hydrolysis and precipitation as calcium pectate.

Method.

A suitable amount of the dried and powdered tissue is extracted for 24 hours at 85° with about 200 cc. of the extracting solution. At the end of that period it is filtered and the residue washed with more solvent. The filtrate and washings are made up to 250 cc. 100 cc. of this extract are gently concentrated to about 25 cc. on a hot plate. When the extracting fluid is oxalic acid, it should be just neutralised before concentration to obviate any possibility of hydrolysis. After cooling, $3\frac{1}{2}$ volumes of 95 % alcohol, acidified with 5 or 6 drops of concentrated hydrochloric acid, are added. From a series of experiments it would appear that the concentration of acid in the alcohol does not affect the yield, as long as the reaction is quite definitely acid. The final concentration of alcohol should be not less than 70 %, and should be kept the same in all estimations. The object of the concentration of the extract is merely to reduce the quantity of alcohol used and to expedite the subsequent filtration. The precipitate is allowed to stand for several hours, and is then filtered off on a fluted filter-paper and washed with acid alcohol until the washings show no trace of oxalate. The filter-paper with the alcohol precipitate is then placed in a beaker and the precipitate dissolved by boiling with about 40 to 50 cc. distilled water. In the case of the ammonium oxalate extracts very dilute ammonia is employed instead of water at this stage. The use of ammonia is necessary because if any salt of pectic acid were present in the tissue, it would now, as a result of the acid-alcohol treatment, be present as free pectic acid, which is insoluble in water but soluble in dilute ammonia. The solution is filtered off, and the filter-paper boiled again, this time with dilute ammonia in the case of aqueous or oxalic acid extracts, and with water in the case of ammonium oxalate extracts. Ammonia is employed in the former cases to ensure that any pectic acid which may have been formed by partial hydrolysis during the concentration or extraction will not be lost. Finally, a further quantity of water is added to the filter-paper, which is then triturated with a stirring rod and boiled. This is filtered, and washed with hot water and hot dilute ammonia. The combined filtrate and washings, usually a little over

100 cc., are treated after cooling with 100 cc. 0·4 % NaOH and allowed to stand overnight. To precipitate the pectin, now in solution as sodium pectate, the calcium gel method is employed. 50 cc. normal acetic acid are added together with an equal volume of 11·1 % calcium chloride and the whole is allowed to boil for at least 5 minutes. It is then filtered as hot as possible through a tared filter-paper—a good Swedish type being the most satisfactory—and the precipitated calcium pectate gel washed with boiling water until the washings are quite free from chloride. As much as 400 cc. of boiling water may be required, but filtration is usually extremely rapid. The precipitate is then dried at 100° and weighed.

The method recently described by Emmett and Carré [1926] resembles the above, which, however, was in use for this work at the time. These workers do not add ammonia when dissolving up the pectin precipitated by alcohol, and in consequence lose any pectic acid which may be present. Also they filter and weigh the calcium pectate gel in a Gooch crucible, a method not found convenient as filtration is slow and washing appears to be unsatisfactory.

Differentiation of extracts.

It is now necessary to consider the specific nature of the pectic substances removed by each of the extracting agents employed, namely water, 0·5 % oxalic acid, and 0·5 % ammonium oxalate. It is generally accepted that three types of pectic substances exist in the plant; protopectin or pectose, the form insoluble in water, which appears to give rise to pectin on hydrolysis, and is often stated, without evidence, to be in combination with cellulose; pectin, the form soluble in water; and pectic acid, insoluble in water, consisting of de-esterified pectin.

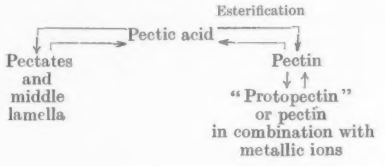
Tutin [1923] questioned the existence of protopectin, stating that the persistent retention of pectin in a form apparently insoluble in water in the tissue of fruits is due to the great difficulty of obtaining by mechanical means really efficient disintegration of the tissue. This is partly true, as the degree of penetration of the extracting fluid depends on the state of division, which may consequently be regarded, up to a point, as a limiting factor. Carré [1925] replied to this criticism, producing evidence which seems conclusive for the presence of some pectic substance insoluble in water. This substance could be removed to a certain extent by very prolonged boiling with water or completely by treatment with dilute acids. She states definitely that this is "pectose" —pectin in combination with cellulose, the pectin of which could be removed completely by boiling for a few hours with acid as dilute as $N/75$ HCl.

In 1925, both Kopaczewski [1925] and Nanji, Paton and Ling [1925] noted that pectin gives, with a solution of an iron salt, a gelatinous precipitate which is insoluble in water. It is but slowly dissolved on prolonged treatment with water at 100°, but is easily soluble in dilute oxalic and hydrochloric acids. These observations caused the latter workers to suggest that pectin exists in a condition insoluble in water in the plant in loose combination with metallic

ions, such as iron. If pectin is precipitated with ferric chloride, the iron content of the precipitate is found to vary very considerably, as iron is carried down by the gel formed. Consequently, it is impossible to determine by simple analysis whether or not simple salt formation has taken place. This is, however, very unlikely, as it would involve the losing of 75 % of the theoretical methoxyl content at least, a change not observed. It seems more likely that the insoluble form is a polymer, consisting of several molecules of the tetramethylpectic acid, associated with one or two partially de-esterified molecules, the free carboxyl groups of which are linked to the metallic ion. Ehrlich [1917] regarded it originally as an insoluble calcium-magnesium salt, but there is evidence from the ash of pectin preparations that it is a mixed calcium-iron salt. Thus it would appear that the insoluble form of pectic substance, hitherto known as "protopectin," is to be regarded as being but the normal form in combination with certain metallic ions as just indicated, and it is this substance that is removed by treatment with 0·5 % oxalic acid or 0·5 % ammonium oxalate.

But it must not be forgotten that this is not the only pectic substance insoluble in water. It is well established that the middle lamella is composed of combined pectic acid. It has been suggested that it is combined with cellulose, but this is very unlikely in the mature plant, as it is easily removed by 0·5 % ammonium oxalate, or by very dilute sodium hydroxide. In addition to its occurrence in the middle lamella, it seems probable from a consideration of the experimental work later to be described that combined pectic acid, *i.e.* pectates, occurs to a much greater degree in some tissues than can be accounted for by supposing that it forms the middle lamella only. This is particularly the case in the rind of the lemon and orange. Traces of free pectic acid have also been found in some tissues. This would also be extracted by 0·5 % ammonium oxalate.

From a consideration of the data above it would seem that pectic acid is the central pectic substance. Which, however, is the precursor, and the mode of its formation, can at present be a matter for conjecture only. Carré [1925] makes the interesting suggestion that the pectic acid may play a useful part as a fixing agent for the methyl alcohol produced as a by-product in metabolism. The relations of these substances are shown in the following scheme:



The solubilities of these substances may be summarised as follows.

Soluble in water—free pectin.

Insoluble in water, but soluble in dilute acids—pectin in combination with metallic ions.

Insoluble in water, soluble in 0·5 % ammonium oxalate—pectin in combination with metallic ions, plus pectic acid, both free and combined.

Therefore in the scheme of extraction followed in this investigation:

A. water—removes free pectin:

B. 0·5 % oxalic acid—removes free pectin, plus pectin in combination with metallic ions:

C. 0·5 % ammonium oxalate—removes free pectin, plus pectin in combination with metallic ions, plus pectic acid, both free and combined.

Consequently, it follows that

A = Free pectin.

$B - A$ = Pectin in combination with metallic ions.

$C - B$ = Pectic acid: free and combined.

The results of this differential estimation of the pectic substance in various samples of leaves, cereals, and fruits are given in Table II. It

Table II. *Distribution of the individual pectic substances in nature.*

	Calcium pectate number*						
	Expressed on dry powder			Expressed on dry powder			
	A	*B*	*C*	*A*	*B* − *A*	*C* − *B*	
Leaves.	Water extract	Ox. acid extract	Amm. ox. extract				Moisture %
I. Sycamore	3·87	5·24	7·90	3·87	1·37	2·66	64·25
II. Alder	1·63	4·12	4·83	1·63	2·49	0·71	82·45
III. Potato	2·10	6·26	7·32	2·10	4·16	1·06	87·96
IV. Sumach	1·82	4·91	5·52	1·82	3·09	0·61	62·91
V. Lilac	2·51	3·70	6·11	2·51	1·19	2·41	66·46
VI. Laurel	2·87	4·52	4·95	2·87	1·65	0·43	77·87
VII. Ivy	3·41	4·96	7·46	3·41	1·55	2·51	70·92
Cereal grains.							
VIII. Barley	0·071	1·02	2·10	0·071	0·95	1·08	12·30
IX. Malt†	0·094	0·158	0·311	0·094	0·06	0·153	5·96
X. Wheat	Nil	0·404	0·606	Nil	0·404	0·202	11·65
XI. Rye	Nil	0·130	1·003	Nil	0·130	0·87	17·55
XII. Maize	Nil	Nil	0·590	Nil	Nil	0·59	11·87
XIII. Oats	Trace	0·43	0·651	Trace	0·43	0·221	14·93
XIV. Rice	Nil	Trace	1·13	Nil	Trace	1·13	13·35
Fruits							
XV. Apple—							
Peel	9·15	11·89	17·44	9·15	2·74	5·55	80·30
Pulp	8·92	11·94	17·63	8·92	3·02	5·69	88·00
Aver. section	8·96	11·92	17·60	8·96	2·96	5·68	87·84
XVI. Orange—							
Yellow rind	7·54	11·09	15·92	7·54	3·55	4·83	73·18
White peel	18·53	20·59	38·75	18·53	2·06	18·16	76·28
Pulp	10·45	12·06	12·40	10·45	1·61	0·34	89·08
Aver. section	14·34	15·50	24·54	14·34	1·15	9·04	84·65
XVII. Lemon—							
Peel	15·92	17·31	35·45	15·92	1·39	18·14	76·62
Pulp	11·31	13·74	14·10	11·31	2·45	0·36	83·39
Aver. section	14·92	15·49	23·31	14·92	0·57	7·82	83·12

* "Calcium pectate number" may be defined as yield of calcium pectate per 100 g. material.

† No. IX was a special research specimen prepared from Barley No. VIII.

should be emphasised here that the results in these tables are all expressed as yield of calcium pectate per 100 g. material. All the figures recorded are the average of at least two, and often more, closely agreeing determinations. Expression of the results as calcium pectate is at present the only sound method, until we know definitely the exact constitution of the substance extracted in each case. The figures for aqueous extract can be expressed if desired as tetramethylpectic acid by the use of the factor 0·9813, which represents the molecular weight of the tetramethyl ester divided by that of calcium pectate. It is by no means certain that free pectin in the plant is in the tetramethyl condition. Norris and Schryver [1925] state that it is uniformly in the tri-esterified condition. Some evidence on this point will appear in a later communication.

Discussion of results.

Leaves. Though a fairly representative selection of leaves was taken, there do not appear to be many points which stand out. Nos. II and III, alder and potato respectively, give very similar figures when expressed on the fresh leaf. Both these plants are comparatively quick growing and sappy in nature as shown by the moisture contents, which are both over 80 %. Whether or not this similarity in content of pectic substances is accidental it must remain for further work to show. There does not appear to be any close similarity between the two representative evergreens selected, ivy and laurel.

Cereal grains. The results show clearly that pectic substances are present in cereal grains only to a small extent—the highest figure recorded for total content being only just over 2 % of the dry weight, in the case of barley. It may be stated that there is practically no free pectin in any cereal, and the pectic substance present is nearly all in the form of pectate. Histological work, to be published elsewhere, shows this to lie almost exclusively in the middle lamella and cell-wall of the aleurone layer. Nos. VIII and IX are interesting, inasmuch as they were special research samples, the malt, No. IX, being obtained from the barley, No. VIII. It would seem that during the process of germination in malting the free pectin increases slightly, probably at the expense of the pectin in combination with metallic ions, while the latter and the pectates decrease enormously. In this connection the work of Tollens [1898], Schöne and Tollens [1901], and of Windisch and Hasse [1901], is very significant. These workers state that during germination an increase of pentosan takes place, and, from the observations mentioned above, there can be little doubt but that this increase is at the expense of the pectic material.

Fruits. It should be noted that the results given under the heading of "average section" were obtained from wedge-shaped slices from the fruits. Simple sections cannot be regarded as giving a true average sample. The main point of interest in the distribution in the fruits examined is the surprisingly high content of pectates in the rind of the orange and the lemon, and the low content of the same in the pulp of these fruits. The free pectin appears to be distributed through the fruits fairly evenly, the region containing the lowest

amount being the pulp. Experiments on the gently expressed juice show that it contains by no means all of the free pectin, and consequently some of this must be regarded also as forming a part of the cell-wall.

SUMMARY.

A method for the differential extraction of the individual pectic substances from plant materials, suitable for routine work, is indicated, and the estimation of each by an improved method as calcium pectate is described. Results are given in the case of certain leaves, cereal grains and fruits.

In conclusion, it is a pleasure to acknowledge the advice and encouragement given by Prof. A. R. Ling.

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LXXVII. ON LACTIC DEHYDROGENASE[1].

A CELL-FREE ENZYME PREPARATION OBTAINED FROM BACTERIA.

By MARJORY STEPHENSON.

From the Biochemical Laboratory, Cambridge.

(*Received March 5th, 1928.*)

Preliminary observations.

During an attempt to correlate the dehydrogenating activity of washed bacterial suspensions of *B. coli* with the number of living bacteria present, the rates of reduction of methylene blue by four hydrogen donators (glucose, succinate, formate and lactate) in the presence of the bacterial suspension were plotted at intervals against the viable count. It was soon found that, though the rates of reduction by the first three donators fell off continuously with the age of the suspension, that of the lactate first fell off, then became stationary and finally rose. This led to a search in the filtrate for a dehydrogenase which should activate lactate in preference to the other three substances. Strong evidence for the existence of this was forthcoming, but it was found that the dehydrogenase was better prepared by allowing thick bacterial suspensions to autolyse at 37°; finally the following method of preparation was adopted.

Preparation of the enzyme.

Ten Petri dishes containing tryptic broth agar (p_H 7·6) with the addition of 0·5 % sodium lactate were thickly sown with a 20-hour culture of *B. coli* and incubated at 37° for 48 hours. The growth was then washed off with water slightly buffered and the suspension centrifuged and washed twice and finally suspended in 80 cc. of $M/2$ phosphate buffer at p_H 7·6 and incubated in a stoppered bottle for 5 or 6 days. The suspension was then centrifuged and the activating power of the supernatant liquid towards the four hydrogen donators tested. In this and all subsequent experiments the following method was adopted. 1 cc. of phosphate buffer p_H 7·6, 1 cc. of 1 in 5000 methylene blue, 1 cc. of water, 1 cc. of $M/20$ donator and 1 cc. of the enzyme preparation were put in a Thunberg tube, which was evacuated and put in a water-bath at 40°, and the reduction time was noted. In subsequent experiments this procedure will be assumed and the donator and reduction time alone recorded.

	Glucose	Succinate	Formate	Lactate
Reduction time	∞	2 hrs.	6 mins.	2¼ hrs.

[1] A short summary of this work appeared in *Chemistry and Industry*, Nov. 11, 1927.

In order to compare the reducing powers of the cell debris and the supernatant liquid respectively, the preparation was again centrifuged and the deposit made up to the original volume with phosphate buffer, and the dehydrogenating power compared with the supernatant liquid.

	Lactate
Reduction time deposit	2 hrs.
,, ,, supernatant liquid	8 mins.

This observation makes it highly improbable that the reducing power of the preparation is vested in the solid phases of the cell. Further experiments showed that the addition of 1 % sodium fluoride to the phosphate buffer in which the cell suspension was autolysed was useful in preventing the deterioration of the preparation through putrefaction, and was found not to alter the properties of the enzyme in any of the particulars described.

Although by repeated centrifuging it is possible to obtain a preparation of lactic dehydrogenase which is, as far as other common donators are concerned, specific for lactate, this is more rapidly effected by filtering through kieselguhr after centrifuging once or twice; by this means, traces of other dehydrogenases, particularly that acting on formate, are held back. For example, an autolysed cell suspension was centrifuged and the supernatant fluid passed twice through kieselguhr on a Büchner funnel; the preparation was almost water-clear. The comparison of the dehydrogenating power of this preparation (IX liquid) with that of the solid material centrifuged from it and diluted till it was of the same activity towards lactate (IX solid) and also with that of a suspension of washed bacteria illustrates the result of the treatment.

	Reduction time of			
	Glucose mins.	Succinate mins.	Formate mins.	Lactate mins.
Cell suspension	15	62	24	8
Prep. IX solid	∞	35	7	6
Prep. IX liquid (after 1st filtration through kieselguhr)	∞	∞	∞	6
,, ,, (after 2nd filtration through kieselguhr)	∞	∞	∞	$8\frac{1}{2}$

The appearance of the preparation at this stage is either very faintly opalescent or even water-clear. Seen under the microscope, using dark ground illumination, the preparation is found to be quite cell-free though very fine remnants of cell debris may still be visible; although these are reduced by centrifuging, it has not been found possible to eliminate the last traces; as stated earlier, however, the activity of the preparation does not seem to be associated with their presence. Passage through a porcelain filter, however, completely inactivates the enzyme. The enzyme may be concentrated by saturation with ammonium sulphate, being totally carried down with the protein precipitate; the total activity is, however, thereby somewhat decreased.

Exp. 30 cc. of the enzyme preparation were saturated with ammonium sulphate at p_H 7·0 and the whole was centrifuged; the supernatant liquid was poured off and the precipitate redissolved in 10 cc. of buffer p_H 7·6.

	Reduction time with lactate
Original preparation	13 mins.
Supernatant liquid	∞
Redissolved precipitate (original conc. 3)	7 mins.

Specificity.

This enzyme reduces lactate; a number of hydrogen donators activated by suspensions of *B. coli* [Quastel and Whetham, 1925, 1, 2] have been tested and found negative; the following are among those tried: glucose, succinate, formate, acetate, glycerol, malate, alanine, ethyl alcohol and acetaldehyde. In their work on associated systems of enzymes in bacterial suspensions, obtained by treatment of the suspensions by various cell poisons, it was found by Quastel and Wooldridge [1927] that the dehydrogenation of lactate was usually associated with that of α-hydroxybutyrate; on their suggestion, this substance was tested with the lactic dehydrogenase and was found to be slowly dehydrogenated. As compared with lactate the process was much slower and it was found necessary to use the α-hydroxybutyrate in ten times the concentration of the lactate, and even then the reduction took about four times as long.

Exp.

Donator	Conc. in Thunberg tube	Reduction time mins.
Lactate	*M*/100	11
Hydroxybutyrate	*M*/5	42
,,	*M*/10	48
,,	*M*/20	68
,,	*M*/40	192

These slow rates suggested that the reduction might be due to traces of lactic acid present as impurity; the α-hydroxybutyric acid was therefore distilled at 1 mm. pressure[1]. The first fraction distilling at about 120° was collected and the distillation stopped when the temperature reached 125°. The distillate and residue were then dissolved and neutralised, and the reduction times of a series of dilutions compared.

Exp.

Hydroxybutyrate. Concentration	*N*/5	*N*/10	*N*/20
	Reduction time		
	mins.	mins.	mins.
Distillate	29	42	63
Residue	33	48	68

These inconsiderable differences do not suggest that the reduction is due to lactate which—if present—should be concentrated in the distillate.

Behaviour towards Clark's indicators.

In order to determine, if possible, the position of the reducing system on the oxidation reduction potential scale, solutions of Clark's series of indicators

[1] For suggesting this procedure and for help in carrying it out I must thank Mrs Needham.

(kindly supplied to me by Dr Dixon) were made in concentrations equivalent to 1/5000 methylene blue and the reduction times with the enzyme and lactate compared.

Indicator	No.	Reduction time	Order of reduction: Lactic dehydrogenase	Order of reduction: Xanthine oxidase	r_H range of indicator
Indigotindisulphonate	1	About 2 hrs.	7	7	7·2–11·2
Indigotintrisulphonate	2	„ 5 „	8	8	8·3–12·3
Indigotintetrasulphonate	3	> 5 „	9	9	10 –14
Methylene blue	3*a*	13½ mins.	—	—	13 –17
1-Naphthol-2-sulphonate-dichloro-indophenol	4	106 „	6	6	15·2–19·2
1-Naphthol-2-sulphonate-indophenol	5	75 „	5	5	15·4–19·4
o-Cresol-2 : 6-dichloro-indophenol	6	12 „	3	4	17·3–21·3
o-Cresolindophenol	7	14 „	4	2	17·7–21·7
2 : 6-Dibromophenolindophenol	8	7½ „	2	3	18·3–22·3
o-Chlorophenolindophenol	9	6 „	1	1	19 –23

It is seen that all the indicators were in time completely reduced and that, with the exception of Nos. 1, 2 and 3, the order closely follows that which would be predicted from the position of the indicators on the r_H scale; the behaviour of xanthine oxidase is appended for comparison [Dixon, 1926]; the r_H range therefore lies outside that of any of these indicators, *i.e.* below 7.

Behaviour towards molecular oxygen.

Whereas the enzyme rapidly transfers hydrogen to the reducing dyes, its behaviour towards oxygen is otherwise. Thus enzyme + buffer + lactate (same quantities as used in the Thunberg tubes), if placed in a small wash-bottle through which a stream of air is passed, produces no pyruvate as shown by the nitroprusside test; if, however, methylene blue be added to the system, rapid oxidation of the lactate occurs and pyruvate is formed. This is shown by first removing the methylene blue by shaking with kieselguhr and filtering and then applying the nitroprusside test.

The observation may be confirmed by a study of actual oxygen uptakes. For this purpose the Barcroft differential manometer was employed with the flat-bottomed cups used in tissue oxidation work, with 40 % potash in the vertical tubes.

Exp.

Fig. 1. Curve 1.

Left-hand cup	Right-hand cup
1 cc. buffer p_H 7·6	1 cc. buffer
1 cc. enzyme	1 cc. enzyme
1 cc. water	1 cc. 0·012*M* sodium lactate

Fig. 1. Curve 2.

Left-hand cup	Right-hand cup
1 cc. buffer with 1/5000 meth. blue	1 cc. buffer with 1/5000 meth. blue
1 cc. enzyme	1 cc. enzyme
1 cc. water	1 cc. 0·012*M* sodium lactate

Fig. 1 shows the absence of uptake in the system enzyme + lactate and the positive uptake when methylene blue is added; the calculated uptake for the change of lactate to pyruvate is shown by the horizontal dotted line at 137 mm.[3]

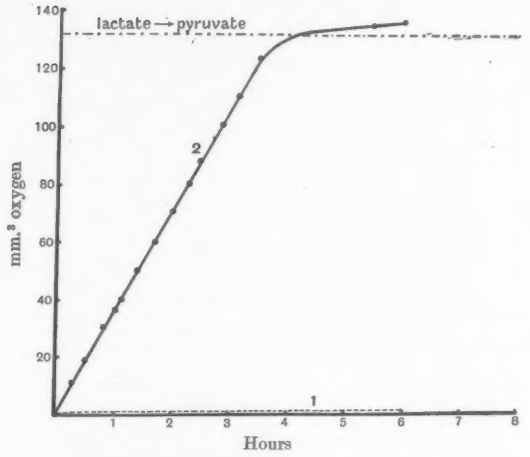

Fig. 1.

– – – – – Oxygen uptake by enzyme + 1 cc. 0·012 *M* sodium lactate.
——— Oxygen uptake of same system + 1 cc. 1/5000 methylene blue.

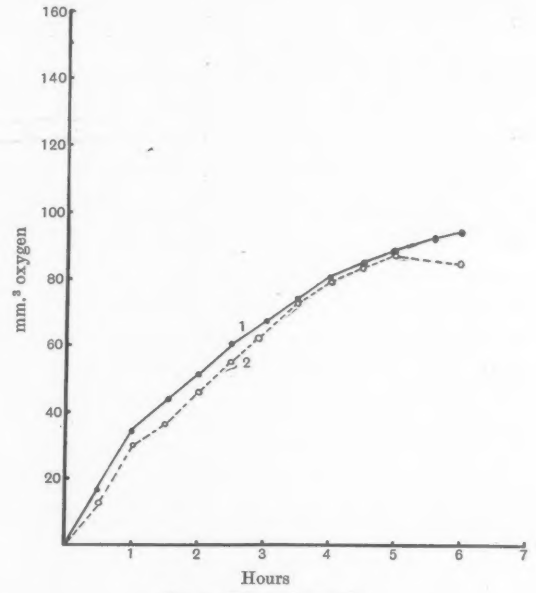

Fig. 2. Effect of cyanide.

•——•——• Oxygen uptake without cyanide. } Enzyme + 1 cc. 0·012 *M* Na lactate + 1 cc.
o– –o– –o Oxygen uptake with cyanide. } 1/5000 methylene blue.

It thus appears that this enzyme is a pure dehydrogenase, that is to say, able to catalyse the transfer of hydrogen to an acceptor already activated but powerless to do so to molecular oxygen except by the aid of an intermediary; this property agrees with its behaviour towards cyanide.

Exp. Fig. 2. Curve 1.

Left-hand cup (1)	Right-hand cup (2)
1 cc. buffer in 1/5000 meth. blue	1 cc. buffer in meth. blue
1 cc. enzyme	1 cc. enzyme
1 cc. water	1 cc. 0·012*M* sodium lactate

Fig. 2. Curve 2.
Same as Curve 1 with 0·001*M* KCN in both cups.

From Fig. 2 it is seen that the system enzyme + methylene blue + lactate is not appreciably inhibited by cyanide.

It is interesting to compare the behaviour of the cell-free lactic dehydrogenase with the same system in the cell suspensions[1]. For this purpose a suspension of washed bacteria (*B. coli*) was prepared in buffer and diluted so as to reduce methylene blue in the presence of lactate in the same time as an equal volume of cell-free enzyme and the oxygen uptake of the two preparations in the presence of lactate compared.

Exp. Fig. 3. Curve 1.

Cup 1	Cup 2
1 cc. buffer	1 cc. buffer
1 cc. bacterial suspension	1 cc. bacterial suspension
1 cc. water	1 cc. 0·012*M* sodium lactate

Fig. 3. Curve 2.

Cup 1	Cup 2
1 cc. buffer in 1/5000 meth. blue	1 cc. buffer in 1/5000 meth. blue
1 cc. enzyme	1 cc. enzyme
1 cc. water	1 cc. 0·012*M* sodium lactate

Fig. 3. Curve 3.

Cup 1	Cup 2
1 cc. buffer in 1/5000 meth. blue	1 cc. buffer in 1/5000 meth. blue
1 cc. bacterial suspension	1 cc. bacterial suspension
1 cc. water	1 cc. 0·012*M* sodium lactate

It is seen that in the case of the washed cell preparation (Curve 1) the uptake is rather more rapid and much more prolonged than with the enzyme (Curve 2), and that it continues long past the point at which the lactate is oxidised to pyruvate. On adding methylene blue to the cell suspension (Curve 3), no acceleration occurs, but a slight retardation and a stoppage slightly higher than in the case of the enzyme preparation + methylene blue, at a point approximating to the oxidation of lactic to pyruvic acid. The washed cell therefore differs in its behaviour towards lactate from the cell-free enzyme in being able to transfer hydrogen to oxygen direct; the addition of methylene blue decreases instead of, as in the case of the enzyme, conferring this power, and would appear to check the uptake near the pyruvic stage.

The action of cyanide on the washed cell system confirms the fact that a component of the system is here present which is absent from the cell-free enzyme.

[1] Comparable with "resting bacteria" employed by Quastel and Whetham [1925, 1].

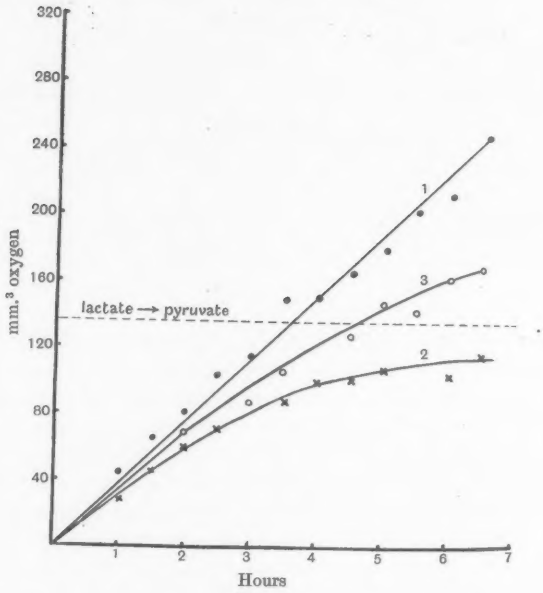

Fig. 3.

Curve 1. •——•——• Cell suspension + lactate.
„ 2. ×——×——× Enzyme + lactate + methylene blue.
„ 3. o——o——o Cell suspension + lactate + methylene blue.

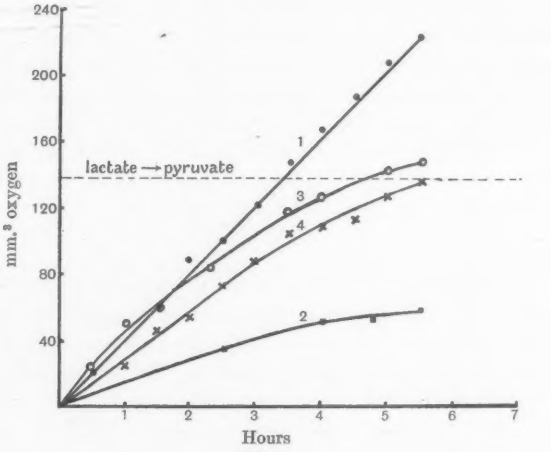

Fig. 4.

Curve 1. •——•——• Cell suspension + lactate.
„ 2. □——□——□ Cell suspension + lactate + cyanide.
„ 3. o——o——o Cell suspension + lactate + methylene blue.
„ 4. ×——×——× Cell suspension + lactate + methylene blue + cyanide.

Exp.

Fig. 4. Curve 1.

Cup 1	Cup 2
1 cc. buffer	1 cc. buffer
1 cc. water	1 cc. bacterial suspension
1 cc. bacterial suspension	1 cc. 0·012*M* sodium lactate

Fig. 4. Curve 2.

Same as Curve 1 + 0·001*M* potassium cyanide.

Fig. 4. Curve 3.

Cup 1	Cup 2
1 cc. buffer in 1/5000 meth. blue	1 cc. buffer in 1/5000 meth. blue
1 cc. bacterial suspension	1 cc. bacterial suspension
1 cc. water	1 cc. water

Fig. 4. Curve 4.

Same as Curve 3 + 0·001*M* potassium cyanide.

It is seen here that 0·001*M* cyanide inhibits the oxygen uptake of the intact cell in the presence of lactate by 80 %; whilst the oxygen uptake of the cell in the presence of methylene blue is only very slightly inhibited by cyanide (3·3 %), thereby resembling the enzyme system. In short, the intact cell possesses a complete oxidation system for lactate which is strongly inhibited by cyanide; the addition of methylene blue to the intact cell cuts down the oxidative mechanism to a point approximating to that of the cell-free enzyme and renders it almost independent of cyanide.

Behaviour of the solid residue after the extraction of the enzyme.

The solid residue after the removal of the enzyme still retains the power actively to dehydrogenate lactate; this power is, however, not specific, formate, for example, being also powerfully activated. The action of fluoride in modifying the power of these various preparations in taking up oxygen needs mention. The presence of fluoride during autolysis appears to have an inhibitive action on the oxygen uptake of the cell residue and it seemed at first as if the same factor might be held accountable for the failure of the enzyme to dehydrogenate to molecular oxygen. As, however, this inability in the enzyme (though not in the residue) exists also in preparations made in the absence of fluoride, this factor cannot be held accountable for the inactivity of the enzyme towards oxygen. An investigation into the action of fluoride in this respect is being undertaken.

Search for the missing factor in the dehydrogenase system.

Since the washed cell possesses some factor, absent from the cell-free dehydrogenase, which enables the hydrogen to be transferred to molecular oxygen it is of interest to discover its nature. The method adopted was to aerate in small wash bottles:

buffer	1 cc.
enzyme	1 cc.
0·05*M* lactate	1 cc.
x	1 cc.

the experiment being controlled by one in which *x* is represented by 1 cc. of

methylene blue (1/5000). Both tubes were aerated at 40° for 3 hours; at the end of that time both contents were separately shaken with kieselguhr to remove the methylene blue from the control tube, filtered, and pyruvate tested for in the filtrate by the nitroprusside test. The search has not yet been successful; during the investigation x has been represented by the following, all of which gave negative results: boiled yeast suspension, boiled suspension of *B. coli*, yeast suspension heated to 65° for 30 minutes, *i.e.* till the dehydrogenases were inactivated to methylene blue, a suspension of *B. coli* similarly treated, nitrate and finally glutathione both in the oxidised and reduced forms.

Comparison with other systems.

These results confirm and extend the views of Fleisch [1924] with regard to the succinic acid dehydrogenase of muscle. Working in a system where washed muscle took the place of bacterial suspensions and succinate replaced lactate he found the oxygen uptake of the system succinate-enzyme totally inhibited by 0·001*M* cyanide and this completely restored—though not at the initial rate—by methylene blue. This led him to postulate a dual enzyme mechanism for the oxidation of succinic acid by oxygen in muscle: (1) a succino-dehydrogenase in the Wieland-Thunberg sense; (2) a catalyst for the activation of oxygen which "may be an independent oxidising enzyme, or it may be iron in the sense of Warburg [1921]." As has been shown, a precisely analogous state of affairs exists in the behaviour of bacterial suspensions towards lactic acid, but in this case the obtaining of a cell-free dehydrogenase unable to reduce molecular oxygen brings additional confirmation to the view of Fleisch on the dual nature of the mechanism, and incidentally assists towards reconciling the opposing views of Wieland and Warburg.

Lactic dehydrogenase differs from another cell-free oxidase, viz. the Schardinger enzyme, which, as Dixon [1927] has shown, reduces not only methylene blue but also molecular oxygen without any intermediate mechanism, the process being independent of cyanide (except at such high concentrations that the whole enzyme is destroyed).

SUMMARY.

1. By autolysing washed bacterial suspensions (*B. coli*) in phosphate buffer p_H 7·6 a dehydrogenase is extracted; by repeated centrifuging and filtration through kieselguhr this may be obtained in clear aqueous solution free from the other dehydrogenases of the cell.

2. This preparation in the presence of methylene blue actively dehydrogenates lactate and also, though much more slowly, α-hydroxybutyrate: the author has not identified any other substrate upon which the enzyme acts.

3. This enzyme differs from the intact cell in its behaviour towards lactate, not only in specificity but in the fact that it cannot reduce molecular oxygen except in the presence of a carrier such as methylene blue.

4. The enzyme is unaffected by cyanide.

I gladly take this opportunity of thanking Sir F. G. Hopkins for his ever-available help and encouragement. For much special assistance and advice I have to thank Dr Malcolm Dixon. My thanks are also due to the Medical Research Council for a full-time grant.

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LXXVIII. THE COLOUR VARIATIONS OF CYANIDIN CHLORIDE AND 3:5:7:3′:4′-PENTAHYDROXY-FLAVYLIUM CHLORIDE AS RELATED TO ACIDITY AND ALKALINITY.

By CHRISTINA MARY FEAR and MAXIMILIAN NIERENSTEIN.

From the Biochemical Laboratory, University of Bristol..

(*Received March 9th, 1928.*)

Much importance has been attached to the colour reactions given by the anthocyanins and anthocyanidins when treated with acids and alkalis [Onslow, 1925], and the pure blue colour given by cyanidin chloride with sodium carbonate has been used as a means of identification of cyanidin chloride from natural as well as synthetical sources [*e.g.* Willstätter and Mallison, 1914; Willstätter and Nolan, 1915; Willstätter, Zechmeister and Kindler, 1924; Pratt and Robinson, 1925]. The positions of methoxy-groups as well as sugar radicals have also been deduced on the basis of the different shades of blue given by these substituted cyanidin derivatives when treated with alkalis [*e.g.* Willstätter and Burdick, 1916; Robertson and Robinson, 1927; Karrer, Widmer *et al.*, 1927].

In the course of other work which has been in progress in this laboratory during the last few years doubt was expressed as to the correctness of the generally accepted 3 : 5 : 7 : 3′ : 4′-pentahydroxyflavylium formula for cyanidin [Nierenstein, 1926], and comparisons were made, therefore, between natural cyanidin chloride from two natural sources (cornflowers and rose petals) and synthetic cyanidin chloride (3 : 5 : 7 : 3′ : 4′-pentahydroxyflavylium chloride). Amongst the many differences noted by Malkin and Nierenstein [1928] it was found that, whereas both natural cyanidin chlorides give a pure cornflower blue, synthetic cyanidin chloride gives under identical conditions a violet colour, which only turns deep blue on the addition of more sodium carbonate. This obviously suggested that the difference was due to the degree of alkalinity used in the reactions and this was confirmed by the use of Sørensen's buffer solutions covering a range of p_H values from 1·04 to 12·97. Thus it was found that the instantaneous production of a pure blue colour took place in the case of cyanidin chloride at p_H 8·04, whereas 3 : 5 : 7 : 3′ : 4′-pentahydroxyflavylium chloride required as high a p_H as 11·57 to give pure blue. Our results also show that the time factor plays an important rôle in the reaction and that different results are obtained if the colour changes are observed instantaneously, after 17 seconds or 5 minutes 17 seconds.

The foregoing observations show, therefore, that the colour changes of anthocyanidins and probably anthocyanins, in presence of acids and especially alkalis, must be standardised before any real value can be attached to them. A knowledge of the p_H values and temperatures, as well as the time of contact with the reagent, is obviously essential for reliable results.

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